

# **THE ROLE OF MYOCARDIAL GLYCOGEN IN THE ISCHAEMIC HEART**

by

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## DECLARATION

I declare that the work included in this thesis was undertaken during my PhD studentship at the Cardiovascular Research Unit, Department of Medicine, University of Edinburgh and written up thereafter. I was the principal contributor to all sections.

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I dedicate this thesis to my parents, Mr Chao Jun De and Mrs Hong Xiu Rong in Beijing, whom I respect and love so much. I would like to thank them for their love and for always having confidence in me.

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## ABSTRACT

More than half of the total mortality from coronary heart disease results from sudden cardiac death, primarily from ventricular fibrillation (VF). The metabolic changes due to ischaemia are believed to play an important role in the genesis of the arrhythmia. Two of the main mechanisms of myocyte cell death in severe ischaemia are inadequate supply of glycolytically produced adenosine triphosphate (ATP) and increased circulating catecholamines (Opie, 1993). Glucose-insulin-potassium (GIK) protects ischaemic myocardium. However, the role of glycolytic ATP is questioned by the results that preconditioned animals have a better recovery although myocardial glycogen was decreased. The precise effect of GIK is still poorly understood. There has been no evidence for the effects of myocardial glycogen on ischaemic noradrenaline (NA) release. Such a study could provide an alternative explanation for the protective effect of myocardial glycogen on VF.

Attention was focused on the effects of myocardial glycogen raised by fasting or GIK infusion on the ischaemic myocardium. Isolated perfused rat hearts were used and retrogradely perfused. Myocardial metabolites and coronary lactate production were measured. The effects of insulin on total anoxia induced NA release were examined. Regional myocardial glycogen levels in the non-ischaemic and ischaemic myocardium of fibrillating and non-fibrillating hearts were also studied. Hearts were freeze clamped at the onset on VF for the measurement of myocardial metabolites. Anaesthetics were necessary to do these studies. However, the effects of anaesthetics on myocardial glycogen levels are unknown and were examined.



Enhanced myocardial glycogen levels obtained by the use of a perfusate containing high glucose (15 mM) and / or insulin and fasting did not reduce the incidence of ischaemia-induced VF. Pre-perfusion of hearts under normoxic conditions with insulin decreased anoxic NA overflow under conditions of low  $K^+$  (3 mM) concentrations. The myocardial glycogen *in situ* did not increase after 24 hours fasting, but after 48 hours fasting it did increase. However, this did not affect the incidence of ischaemia-induced VF *in vitro*. The myocardial glycogen levels raised by fasting were normalised within 10 minutes of isolated perfusion with 5.5 mM glucose. Pentobarbitone, which gave the highest myocardial glycogen levels, can be used for glycogen studies *in vitro*, provided that hypoxia is prevented during the induction of the anaesthesia.

In conclusion: The results in this thesis suggest that the method used to increase myocardial glycogen concentration is more important than the glycogen level itself in protecting the heart against ischaemia.

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# **CHAPTER 1**

## **Introduction**

More than half of the total mortality from coronary heart disease results from sudden cardiac death. The most common rhythm changes are ventricular tachycardia (VT) and ventricular fibrillation (VF). The underlying mechanism of the arrhythmias is that the ischaemia causes various metabolic changes including decreased pH; and increased extracellular potassium levels due to enhanced efflux. The efflux is believed to be the result of increased conductance, intracellular accumulation of permeate anions such as lactate, and reduced activity of the  $\text{Na}^+/\text{K}^+$  ATPase, which result in an alteration in action potentials (Hirche *et al.*, 1980).

In addition, there is a progressive release of noradrenaline (NA) from the myocardial adrenergic nerve terminals within the first hour after the onset of ischaemia (Abrahamsson *et al.*, 1984). This overflow of noradrenaline is believed to play an important role in the development of arrhythmias (Rona, 1985), and to contribute to the increasing calcium influx into the myocyte and the progression of ischaemic cell injury and cell death. However, the mechanisms responsible for changing from a reversible to an irreversible state of myocardial injury are still not known with certainty. Improved understanding of the metabolic changes in the disease is crucially important for effective prevention and treatment.

## **1.1 MYOCARDIAL GLYCOGEN AND ISCHAEMIA**

The heart has relatively poor glycolytic ability and there are large numbers of mitochondria ensuring that metabolism is essentially always aerobic to produce adenosine triphosphate (ATP) (Opie, 1986). It is therefore understandable that under physiological conditions, for maximal oxidative ATP production, fatty acids are the preferred exogenous substrate to supply 60 - 80% of the energy demands (Liedtke,

1981; Neely and Morgan, 1974). The remaining 20 - 40% is derived from the oxidation of glucose, lactate and ketone bodies. In fact only a negligible fraction of its energy comes from anaerobic glycolysis (Morgan *et al.*, 1961). Under anaerobic conditions, more myocardial energy does come from glycolysis, and glycogen is an essential substrate (Opie, 1986). The crucial importance of glycolysis is to provide ATP in the absence of O<sub>2</sub>. Recently, increased myocardial glycogen has been claimed to protect the heart from ischaemic damage both clinically and experimentally (Lazar, 1994; Svedjeholm *et al.*, 1995). However, under very severe ischaemia, glucose infusion appears to promote rather than prevent arrhythmogenesis. (Liedtke *et al.*, 1976; Russell, and Oliver, 1977). Further investigation is needed in order to understand the precise mechanism of the protective effects of glycogen on ischaemic myocardium.

### 1.1.1 Regulation of Glycogen and Glycolysis

#### Glucose Transport

Glucose transport across the myocardial cell membrane is mainly through passive uptake and by an energy dependent transport system.

Glucose uptake works in the direction of the glucose gradient. The glucose can be taken up by the isolated perfused rat heart at glucose concentrations 0.8 - 1.2 mM. The dog or human heart *in situ* does not extract glucose below an arterial glucose threshold of 3.3 mM (Opie, 1968).

Glucose transport is energy and insulin dependent. Normally, transport of glucose across the myocardial cell membrane is the rate-limiting step in myocardial glucose metabolism (Manchester *et al.*, 1994; Stephens and Pilch, 1995).



## Insulin

The central role of insulin in regulating glucose metabolism has long been recognised. Insulin decreases the normal arterial glucose uptake threshold from 3.3 to 0.6 mM in dogs. The threshold is abnormally high in untreated diabetic dogs and humans (Opie, 1968). Insulin increases the maximal rate of glucose transport from 3.2 - fold in muscle to 30 - fold in adipocytes (Sternlicht *et al.*, 1988; Cushman and Wardzala, 1980; Suzuki and Kono, 1980). Insulin increases membrane glucose transport rapidly, within one to two minutes, with a maximal effect in 15 to 20 minutes. Many tissues, including liver, maintain glucose uptake independent of insulin concentrations (Hertz *et al.*, 1981). In clinical and experimental situations, insulin can improve cardiac pump function. This effect is either by sympathetic stimulation (Rowe *et al.*, 1981; Liang *et al.*, 1982) or by a direct inotropic effect (Rieker *et al.*, 1975; Lee and Downing, 1976).

Das (1973) has reported that in the male Sprague-Dawley rat Langendorff preparation, insulin increases all intermediates up to glycogen, but does not activate hexokinase. The report concludes that insulin increases glycogen synthesis possibly by acting on cell membrane insulin-accelerated glucose transport.

An increase in plasma glucose concentration is the most important physiological regulator of insulin secretion from the pancreas. An *in vitro* study has indicated that the secretion is also affected by the plasma potassium level (Cox *et al.*, 1978), which is understandable as the secretion of insulin is mediated by  $K^+$ -induced depolarisation. Perfusing the pancreas with a raised potassium concentration has been shown to cause increased insulin release (Gomez and Curry, 1973). On the other hand, lowered potassium concentration causes a decline in glucose tolerance associated with impaired insulin secretion (Rowe *et al.*, 1980).

The effects of insulin on cardiac muscle *in vitro* are not exactly the same as *in vivo*. The glucose uptake can be increased in the perfused heart by insulin but only under low work load situations (Nguyen *et al.*, 1990; Taegtmeyer *et al.*, 1980).

The isolated rat heart preparation contains tissue-bound insulin on removal from the body. A pre-perfusion of about 30 minutes is required to reduce glucose transport to a basal level (Zachariah, 1961; Mansford, 1967).

### Starvation

When an animal is deprived of food, it has an over-riding need to maintain blood glucose levels. The biochemical pathways of major importance in the fasting state are glycogenolysis, gluconeogenesis, ketogenesis and ketone body utilisation.

During periods of starvation, the maintenance of plasma glucose levels is critically important to the organism. In the non-ketotic state, the energy needs of the brain can only be met by glucose, and its absence results in the death of central nervous system tissues. The glucose pool can provide only 15 to 20 g and hepatic glycogen averages 70 g glucose in the adult. Together they can only provide glucose for less than 8 hours supply. Thus, gluconeogenesis is important for the maintenance of post-absorptive plasma glucose concentration and becomes the sole source of glucose production beyond a 24 to 48 hour fast. Only the liver and kidneys (not the heart) contain glucose-6-phosphatase, the enzyme necessary for the release of glucose into the circulation, and the liver with its significant amount of glycogen is the immediate source of blood glucose during fasting. Starvation is associated with a decline in insulin and a rise in glucagon concentrations, which result in increased rates of gluconeogenesis. The low plasma insulin concentrations allow glucose to be used less by adipose tissues and thereby reduce re-esterification and enhance lipolysis. The free fatty acids (FFA) are thus more available for use as an oxidative fuel during starvation.

It has been reported that prolonged fasting in rats increases the myocardial glycogen content (Freminet *et al.*, 1984; Gannon and Nuttall, 1984; Williamson, 1962), which has been shown to be due to increasing fatty acids and lactate in the blood, although glucose is still taken up. In consequence, glycogen is formed and conserved (Opie, 1968 and 1969; Scheuer, 1967).

A species comparison study in male animals (12 week old rat and guinea-pigs) by Freminet *et al.*, (1984) has shown that myocardial glycogen levels in male Sprague-Dawley or Wistar rats were increased significantly after 48 hours fasting, but did not vary in guinea-pigs. In female Wistar rats, after 24 or 48 hours fasting, cardiac glycogen was either unchanged (Amall *et al.*, 1988) or decreased (Schaefer *et al.*, 1985). Schneider and Taegtmeier (1991) reported that in male Sprague-Dawley rats (300 - 350 g), the myocardial glycogen content rose by 25% after 16 hours overnight fasting.

Poland *et al.* (1982) found one day fasting elevates cardiac glycogen in one year old and aged (24 - 30 month old) rats, but found no significant increase in the four month old group. The plasma glucose did not decrease significantly except in one year old rats. The plasma FFA levels were increased in every group after fasting. Menahan and Sobocinski (1983) reported that in 6 - 8 week old Sprague-Dawley rats the myocardial glycogen content remained unchanged after 24 and 48 hours fasting although there was a progressive increase in plasma FFA with starvation.

The influence of hormones on myocardial glycogen metabolism in fed and fasted rats has been reported by Conlee and Tipton (1975). Hypophysectomy eliminates the glycogen increase that occurs from fasting in normal animals while insulin deficiency leads to elevated glycogen stores in both fed and fasted animals. They concluded that adrenal hormones exert a stabilising effect on myocardial glycogen levels in fed animals but are not necessary for synthesis to occur.

Fasting is one of the common causes of hypoglycaemia and hypokalemia. Both factors, leading to a state characterised by low insulin levels and raised plasma FFA and ketosis, can lead to elevated glycogen stores in both fed (i.e. as diabetic) and fasted conditions.

Fasting is commonly used to increase myocardial glycogen experimentally. However, the effects of fasting on myocardial glycogen can vary with fasting duration, animal species, gender or age. In addition to the factors highlighted above, the diurnal effect on myocardial glycogen should also be taken into consideration.

### **Diurnal Effects**

The concentration of cardiac glycogen in the rat has a diurnal rhythm (Asimakis, 1996; Segel *et al.*, 1975). Daw and colleagues (1969) reported that the myocardial glycogen levels in the Sprague-Dawley rats were 20% higher in the morning than in the evening. They believed that the changes were related to the diurnal fluctuations in plasma corticosteroid concentrations.

### **Regulation of Glycolysis**

The glycolytic reactions are the same in the presence of  $O_2$  as in its absence, except that the flow through the pathway and end products that are formed differ. When  $O_2$  is in short supply, re-oxidation of NADH formed from  $NAD^+$  during glycolysis is impaired, NADH is therefore re-oxidised by coupling it to the reduction of pyruvate to lactate, and  $NAD^+$  so formed allows further glycolysis to proceed.

The three principal enzymes controlling glycolytic rate are hexokinase (HK), phosphofructokinase (PFK) and pyruvate kinase. HK has low  $K_m$  (the substrate concentration that produces half-maximal velocity) for glucose, reflecting its function to ensure a supply of glucose for the muscle even in the presence of low blood glucose

concentrations. HK catalyses an irreversible reaction. PFK, which catalyses the conversion of fructose-6-phosphate to fructose-1,6-diphosphate, plays a major role in the regulation of the rate of glycolysis after cellular entry of glucose (Opie, 1971). It is modulated by cytoplasmic ATP levels. High concentrations of ATP depress PFK activity (Neely and Morgan, 1974). The optimal PFK activity occurs at pH 8 (Scheuer and Berry, 1967). Direct inhibitory effects of fatty acids on glycolytic enzymes in the heart have been reported by Neely and Morgan (1974), but it is doubtful whether this can happen in myocardium under physiological conditions (Van der Vusse *et al.*, 1982).

**Summary:** The transport of glucose is mainly through a glucose transporter and is glucose concentration and insulin dependent (Danforth *et al.*, 1962; Morgan *et al.*, 1965). Both insulin and fasting can alter myocardial glycogen levels. These changes in myocardial glycogen metabolism are the results of a synergistic relationship between a variety of hormonal and nutritional factors.

### 1.1.2 Energy Supply During Ischaemia

The immediate effect of ischaemia in the myocardium is a decrease in oxygen tension, which causes a shift from oxidative to glycolytic energy supply (Drake-Holland and Noble, 1983; Lopaschuk and Stanley, 1997).

To provide the same amount of high energy phosphate, 19 times more glucose is needed in glycolysis than through the citric acid cycle. It proceeds rapidly for a short time under anaerobic conditions. Animal studies have demonstrated that the rapid increase in the intracellular  $\text{NADH} / \text{NAD}^+$  ratio causes inhibition of  $\beta$ -oxidation of intracellular fatty acids (Opie, 1969) after hypoxia (lack of oxygen) and ischaemia, resulting in the accumulation of fatty acid intermediates (acyl-CoA and acylcarnitine). These metabolites

are capable of inhibiting further fatty acid metabolism despite restoration of coronary blood flow (Folts *et al.*, 1978; Katz and Messine, 1981).

Glucose uptake by the heart is dependent on coronary blood flow. The uptake is increased with moderate ischaemia but decreases with severe ischaemia when glucose delivery to the myocardium is very low. However, ischaemia consistently causes a large increase in the extraction of glucose from the blood (Stanley *et al.*, 1992).

In the dog, accelerated myocardial glycolysis appears less than 10 seconds after perfusion has stopped (Jones *et al.*, 1976). This stimulation of anaerobic metabolism was considered to improve the energy balance and to be an important survival mechanism (Weissler *et al.*, 1968). However, ischaemia also results in a switch from lactate uptake by the heart to lactate production (Neely and Morgan, 1974). During severe ischaemia, high glycolytic rates, which cause lactate accumulation and decreases intracellular pH, may actually contribute to ischaemic injury (Neely and Grotyohann, 1984). Increased lactate is also associated with a rise in cytosolic NADH which inhibits glycolysis within under-perfused ischaemic myocardium and reduces anaerobic ATP production (Neely *et al.*, 1975, Rovetto and Neely, 1979). Within 30 seconds after the onset of ischaemia, the metabolic flux through glycolysis has become marked as compared to the normal state, but a few (2 - 3) minutes later, the rate of glycolysis is only one quarter of the maximum value. However, this less effective glycolysis will still continue for about 30 minutes in the area of total ischaemia (Jones *et al.*, 1976).

Ischaemia causes a marked depletion of creatine phosphate (CP) and ATP. The function of CP is to act as a store of high-energy phosphate to maintain ATP concentrations in muscle, when ATP is being rapidly utilised as a source of energy for muscular contraction. The myocardial CP level falls by over 80% within 2 to 3 minutes after ischaemia, with ATP content declining more slowly (Alpert, 1989). CP is therefore a more

sensitive parameter than ATP to indicate an imbalance between energy supply and demand. To monitor ATP with CP together can provide more detailed information about myocardial energy changes.

Although anaerobic glycolysis cannot produce enough energy (ATP) for the ischaemic myocardium to contract, it can keep the muscle cells alive for 30 - 60 minutes (Opie, 1975).

### **1.1.3 Ischaemia-induced Arrhythmias and Glycogen**

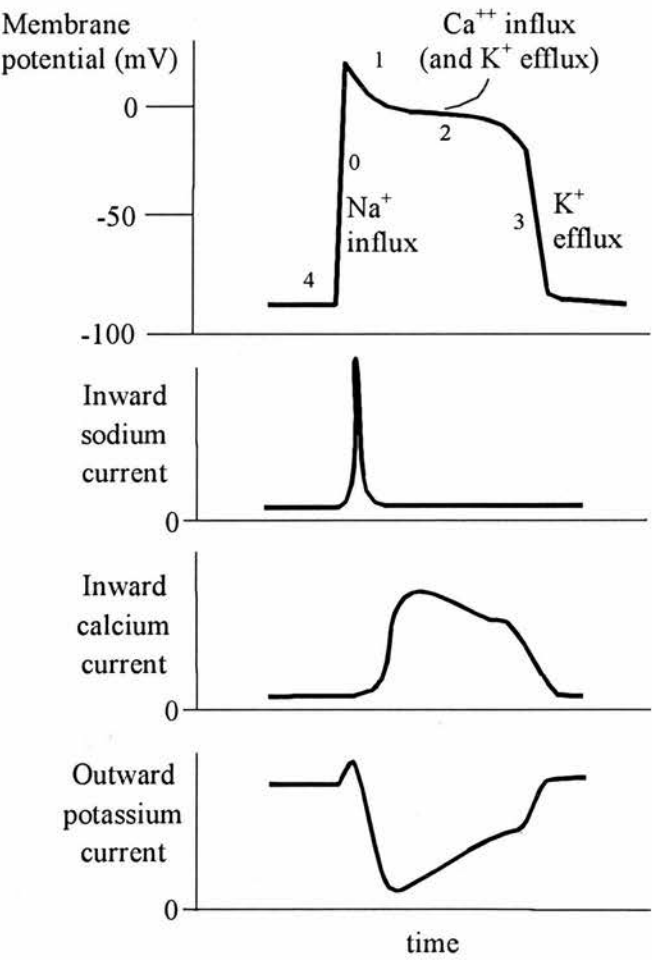
#### **Ischaemia-induced Arrhythmias**

The interior of the resting cardiac ventricular cell is electrically negative relative to the exterior, due to active extrusion of sodium ions which maintain a transmembrane voltage difference of approximately -90 mV. Reduction of the voltage difference to threshold of -60 mV triggers a self-perpetuating action potential. The general electrical activity of cardiac ventricular cell is summarized in Fig. 1.1 and 1.2.

In global ischaemia of intact rabbit and canine hearts, the transmembrane action potential (AP) is decreased in amplitude, and the duration of AP is shortened within a few minutes until cell became unresponsive after 15 - 26 minutes. Similar results were found in ischaemic cells during acute regional ischaemia in porcine and canine hearts. However, the dog hearts were found to have less pronounced changes (Janse and Kleber, 1981). The mechanism for the species difference needs further investigation. The precise mechanism of the AP changes during ischaemia is still in debate.

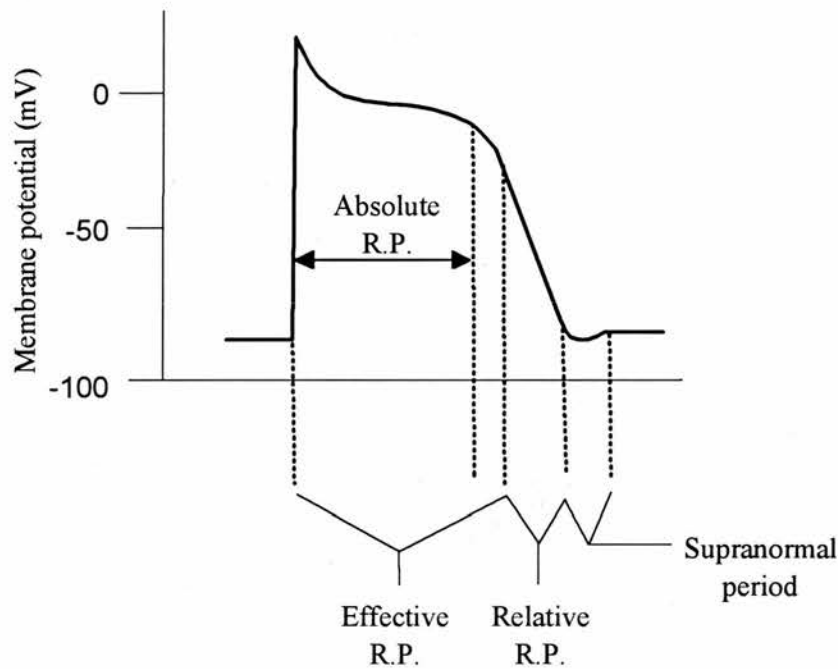
**Fig. 1.1** Schematic representation of a ventricular myocyte action potential (AP), and relative net ion currents for  $\text{Na}^+$ ,  $\text{Ca}^{++}$ , and  $\text{K}^+$ .

The action potential has five parts: Following depolarization, fast  $\text{Na}^+$  influx results in the rapid upstroke of phase 0; a transient outward potassium current is responsible for early repolarization during phase 1; slow  $\text{Ca}^{++}$  influx (and relatively low  $\text{K}^+$  efflux) results in the plateau of phase 2; final rapid repolarization is largely due to  $\text{K}^+$  efflux during phase 3. The resting potential is represented by phase 4 of the AP, the diastole during which the resting transmembrane voltage difference remains until the cell is once again stimulated.





**Fig. 1.2** Refractory periods (RP) of the ventricular myocyte. During the absolute refractory period (ARP), the cell is unexcitable to stimulation. The effective refractory period includes a brief period beyond the ARP during which stimulation produces a localized depolarization that does not propagate. During the relative refractory period, stimulation produces a weak action potential (AP) that propagates, but more slowly than usual. During the supranormal period, a weaker than normal stimulus can trigger an AP.



Experimental evidence has shown that the heart is particularly vulnerable to VF during the first 30 minutes after coronary occlusion. The acute phase of myocardial ischaemia is associated with a high incidence of life threatening ventricular arrhythmias (Janse, 1989). Similar phases of early ischaemia are believed to occur in humans, and can lead to sudden arrhythmic death (Janse, 1986; Surawicz, 1985).

These early arrhythmias occur in two distinct phases (Kaplinsky *et al.*, 1979). The first phase develops between 2 - 10 minutes after onset of ischaemia. The second phase occurs between about 12 and 20 minutes after onset of ischaemia. 76% of the immediate or first phase ventricular arrhythmias are initiated and maintained by a re-

entrant mechanism (Janse and Wit, 1989; Pogwizd and Corr, 1987; Kaplinsky *et al.*, 1979).

The severity of ischaemia can be affected by the status of collateral circulation. It is known that animals with a better collateral coronary supply are less likely to succumb to lethal arrhythmias than those devoid of collaterals. In a dog study, Bolli and colleagues reported that the residual coronary flow of > 8% of normal, when compared with < 8%, substantially reduced the incidence of VT (17% vs. 87%) and VF (0% vs. 57%). This may indicate that residual flow (ischaemia severity) and species differences (collateral coronary supply differences) affect ATP production from anaerobic glycolysis during ischaemia.

In the rat heart, the incidence of VF depends on the size of the ischaemic zone. The peak incidence of VF occurs when the ischaemic zone is 50% of the ventricular weight (Ridley *et al.*, 1992).

Another critical factor in the genesis of early ischaemic and reperfusion VF is the rapid development of electrophysiological inhomogeneities within regionally ischaemic areas of the myocardium (Janse, 1986; Russell, 1981). Russell and colleagues (1986) explored the possibility that these electrophysiological inhomogeneities could to a large extent be determined by similar inhomogeneities of glucose-glycogen utilisation during early ischaemia. They analysed patterns of conduction, glycogen, lactate and high energy phosphate distribution in dog hearts developing early ischaemia-induced ventricular arrhythmias and fibrillation. The inhomogeneities of conduction within ischaemic areas were accompanied by marked inhomogeneities of glycogen, lactate and ATP distributions. They found that inhomogeneities of glycogen distribution were greater in hearts that fibrillated than in those that did not.

An excess of FFA could also increase the risk of ischaemia-induced arrhythmias. Oliver and colleagues (Kurien *et al.*, 1969) first reported that elevation of plasma FFA through activation of lipoprotein lipase was associated with the development of serious ventricular arrhythmias in dogs with acute myocardial infarction. Furthermore, blocking FFA release from triglyceride (TG) and adipose tissue has protective effects on ventricular arrhythmias during myocardial infarction. They concluded that an excess of FFA can, by itself, be arrhythmogenic (Rowe *et al.*, 1975; Oliver and Opie, 1994). Whether this is due to a direct effect of FFA on electrophysiological parameters or whether it is due to an pro-ischaemic effect is not certain. The experimental results were also inconsistent (Riemersma; 1979, Opie *et al.*, 1971a). A similar model was used by Riemersma (1979) by infusion of FFA salts bound to albumin into the blood. The result failed to precipitate ventricular fibrillation.

Recently more emphasis has been placed on the oxidative fate of substrates for myocardial metabolism during ischaemia or recovery from ischaemia. Several anti-ischaemic agents (eg. carnitine, ranolazine) inhibited fatty acid oxidation, and increased glucose oxidation as well (Stanley *et al.*, 1997; McCormack *et al.*, 1996). Whether they also reduce the incidence of the arrhythmias remains to be seen.

The evidence suggest that acute metabolic changes within ischaemic myocardium may play an important role in the genesis of the arrhythmias. One of the modifications which can be made is to enhance the availability of myocardial glycogen, which is the main energy source during ischaemia. This has shown a protective effect under certain conditions.

### The Role of Glycogen in Ischaemic Heart

That sugar has a protective effect on diseased hearts was known long ago. In 1912 cane sugar administered orally was recommended by Goulston for treating certain forms of heart disease (Goulston, 1912). The anti-arrhythmic effects of glucose or glucose-insulin-potassium (GIK) regimes were first demonstrated during acute myocardial infarction more than 30 years ago by Sodi-Pallares and colleagues (1963). They attributed the benefit to "polarising" effects on the ischaemic cell membrane (i.e. increased negative resting membrane potential).

It is known that AP duration has been shown to be dependent on glycolytic ATP (McDonald *et al.*, 1971; McDonald and MacLeod, 1971). McDonald and MacLeod (1972) demonstrated that in anoxic ventricular muscle of guinea pig, incubation in 50 mM glucose medium has a normal AP duration and overshoot. In 5 mM glucose medium the AP duration was severely reduced with only a small decline in overshoot. In the absence of glucose the AP duration was further reduced and the amplitude was depressed by about 35 mV. Re-incubation in 50 mM glucose medium quickly restored both duration and overshoot. They concluded that the maintenance of optimal cardiac muscle glycolytic activity appears to be of the utmost importance, particularly where there is the possibility of inadequate oxygen supply. Insulin was not used in these experiments.

Scheuer and Stezoski (1970) have demonstrated a protective effect of GIK on isolated perfused anoxic rat hearts. In coronary occlusion experiments similar findings were made (Maroko *et al.*, 1972; Ahmed *et al.*, 1978). Opie's team (1975, 1976) reported the protective effects of enhanced myocardial glycogen against myocardial ischaemic injury in the regionally ischaemic dog and baboon hearts. The results from the ischaemic dog study by Russell and colleagues (1986) also supported these findings. They reported that increased endogenous myocardial glycogen stores might be able to protect the heart against early ischaemia-induced arrhythmias. They also found significantly lower myocardial glycogen stores in both ischaemic and non-ischaemic areas of dog

hearts that developed early ischaemic ventricular fibrillation in comparison to areas from non-fibrillating animals. This was despite comparable levels of collateral flow, tissue lactate and ATP. They suggested that hearts with higher pre-ischaemic glycogen contents may be less predisposed to fibrillate. Why some dogs had high myocardial glycogen levels was not examined by them.

The myocardial glycogen content can be increased more than 3-fold by overnight GIK infusion in man (Lolley *et al.*, 1985) and more than doubled within one hour experimentally (Opie *et al.*, 1975). It has been shown that myocardial glycogen loading prior to cardiac surgery can reduce the incidence of pre-operative ventricular arrhythmias (Lolley *et al.*, 1985). GIK infusion has also shown beneficial effects on patients after cardiac surgery (de Villalobos and Taegtmeyer 1995). Insulin alone improves the functional recovery of the perfused rat heart and of diabetic patients subjected to ischaemia (Muller *et al.*, 1978).

More powerful protection against early ischaemia-induced arrhythmias may be achieved by using  $\beta$ -adrenoceptor blockade and by chronic cardiac denervation (Hjalmarson *et al.*, 1981; Ebert *et al.*, 1970).

Certain  $\beta$ -adrenoceptor antagonists, e.g. propranolol; class 1 anti-arrhythmic agents are characterised by their ability to block the fast inward sodium current in cardiac ventricular muscle tissue. It is also known that propranolol only induces  $\text{Na}^+$  channel block at the top end of its therapeutic dose range. The fast channel can give rise to the development of malignant arrhythmias (Opie, 1978).

The chronic sympathetic denervation either surgically or chemically (pretreatment with 6-hydroxydopamine or  $\alpha$ -methylmetatyrosine) with a substantial reduction of cardiac NA content (<10% of normal). This is associated with a reduction in the severity of ischaemic or reperfusion arrhythmias and ischaemic damage of myocardium (Martin and

Meesmann, 1985; Jones *et al.*, 1978; Schwartz and Zuanetti, 1988). Schwartz *et al.* (1985) reported a significant reduction (from 22% to 3.6%) in the incidence of sudden cardiac death in patients having left stellectomy after acute myocardial infarction over 5-year follow-up. The acute surgical sympathectomy, on the other hand, is usually less potent or ineffective (Schwartz, *et al.*, 1976; Elson, *et al.*, 1981; Euler, *et al.*, 1985). These studies suggest the importance of myocardial catecholamine levels, and this also associated an increased myocardial glycogen levels (Daugherty, *et al.*, 1986). Catecholamine is well known to stimulate glycolysis through the activation of c-AMP.

The Langendorff perfused guinea pig hearts study also demonstrated that catecholamine depletion increased myocardial glycogen levels (Culling, *et al.*, 1984).

### **The Mechanism of the Beneficial Effects of GIK**

The initial idea was that GIK, as a "polarising solution", can prevent intracellular  $K^+$  loss by stimulating the sodium pump. Insulin prevents glycogenolysis and lypolysis, and glucose prevents the accumulation of fatty acids. Both promote glycogen deposition in the myocardium. The transport of glucose across the cell membrane is accompanied by  $K^+$ . Potassium was therefore added to prevent systemic hypokalemia (Hoekenga *et al.*, 1988; McDaniel *et al.*, 1985; Bicknell and Opie, 1978).

ATP-sensitive  $K^+$  ( $K_{ATP}$ ) channels are activated when glycolytic ATP concentration falls below a critical level, and have been implicated as a possible cause of the marked increase in cellular  $K^+$  efflux during ischaemia. This coincides with the onset of ventricular arrhythmias. Weiss and Lamp (1989) demonstrated that ATP derived from glycolysis preferentially inhibited the  $K_{ATP}$  channel. This evidence supports the view that GIK may play an important role in keeping the membrane stable during ischaemia.

Despite these encouraging results, the precise metabolic effects of GIK during ischaemia remain poorly understood. The experiments both in humans and animals have given inconsistent results (de Villalobos and Taegtmeyer 1995). The origin of the contradiction is the dual role of glycogen in ischaemic heart. A high level of glycogen is the major source of energy in the ischaemic tissue required to maintain cardiac cell integrity and ionic homeostasis. However, glycogenolysis can result in acidification of the intracellular medium, and lead to disturbances of osmolarity possibly other toxic catabolites that reduces cell viability.

Neely's team reported that increased anaerobic glycolysis during early ischaemia is quickly inhibited by intracellular acidosis (Neely *et al.*, 1975; Rovetto and Neely, 1979). Nevertheless, the rate of glycolysis is not entirely reduced to basal levels, and perhaps slightly higher tissue ATP levels can therefore still be maintained.

Both Neely and Oliver's team have found that, under very severe ischaemic injury, glucose infusion appears to promote rather than prevent arrhythmogenesis. They conclude that this may be a result of earlier inhibition of glucose utilisation during very severe ischaemia due to more rapid development of intracellular acidosis (Liedtke *et al.*, 1976; Russell and Oliver, 1977). Of course in many clinical studies the extent of (collateral) coronary circulation is unknown in the patient receiving GIK.

## **1.2 MYOCARDIAL NORADRENALINE**

Both in patients and in animal models of ischaemia or anoxia, an association has been shown between myocardial infarction and an increase in plasma and urine noradrenaline levels (Richardson, 1963; Valori *et al.*, 1967; McDonald *et al.*, 1969; Jewitt *et al.*, 1969; Nadeau and DeChamplain, 1979; Goldskin, 1981; Karlsberg *et al.*, 1979; Lammerant *et al.*, 1966). It has also been shown that unusually high levels of catecholamines in the



blood, accompanied by a depletion of endogenous noradrenaline stores in the infarcted myocardial area (Mathes and Gudbjarnason, 1971), cause functional and morphological changes in the heart (Valori *et al.*, 1967; Singal *et al.*, 1982; Rona *et al.*, 1963), arrhythmias (Jequier and Perret, 1970; Singal *et al.*, 1985) and sudden death. Suppressing adrenergic activity by using  $\alpha$  and  $\beta$ -blockade can modulate the effects of noradrenaline released and effectively reduce the risk of sudden cardiac deaths (Oliver, 1985) and protect the heart against ischaemic damage and ventricular arrhythmias (Corr *et al.*, 1986; Norris *et al.*, 1984; Peter *et al.*, 1978).

### 1.2.1 Physiology of Sympathetic Nerve Terminal

The mean levels of catecholamines in human plasma obtained from resting subjects are 1 to 1.5 pmol ml<sup>-1</sup> for noradrenaline and about 0.25 pmol ml<sup>-1</sup> for adrenaline and dopamine, with slight variations depending on the method used for assay (Sahai and Ganguly, 1991). In large experimental animals, plasma catecholamine levels are similar to those in humans (Bühler *et al.*, 1978). In early studies, the values in rat blood obtained after decapitation were extraordinarily high, but when suitable precautions were taken to avoid stress, low values, similar to those in humans, are obtained (Chiueh and Kopin, 1978; Benedict *et al.*, 1978; Micalizzi and Pals, 1979). Decapitation elevates levels of adrenaline most (hundred-fold increases or more) and dopamine least (Kvetnansky *et al.*, 1978; Bühler *et al.*, 1978).

Some basic aspects of the cardiac sympathetic nervous system, noradrenaline synthesis, storage, release, and removal, are briefly summarised here in order to fully understand the role of sympathetic nervous system during ischaemia.



### Noradrenaline Synthesis

The rate-limiting reaction is mediated by tyrosine hydroxylase which is inhibited by the end-product, noradrenaline. The enzyme is found only in catecholamine-containing cells. Dopamine is formed in the cytosol, it is then rapidly transported into secretory vesicles by an ATP-driven carrier-mediated process. Once within the vesicles dopamine is converted to noradrenaline by the enzyme dopamine  $\beta$ -hydroxylase (DBH). The conversion of dopamine to noradrenaline within the vesicles has a  $t_{1/2}$  of approximately 6 hours. The rate of conversion is dependent on substrate availability (Corcoran *et al.*, 1984).

Noradrenaline turnover time is generally about 5 - 15 hours, but it becomes much shorter if sympathetic nerve activity is increased. Under normal circumstances the rate of synthesis closely matches the rate of release, so that the noradrenaline content of tissues is constant regardless of how fast it is being released.

### Noradrenaline Storage

Most of the noradrenaline in nerve terminals is contained in vesicles at high concentration of 0.3 - 1.0 M together with ATP (NA : ATP about 1:4 molar ratio within the vesicles). Under normal circumstances, due to monoamine oxidase activity in nerve terminals, only a little is free in the cytoplasm.

### Noradrenaline Release

Exocytotic noradrenaline release is  $\text{Ca}^{++}$ -mediated and is triggered by depolarisation of the nerve terminal membrane (Winkler, 1988). This can be achieved experimentally by increasing the extracellular potassium concentration (a propagation of an action potential is not essential for release), which opens calcium channels in the nerve terminal, and promotes the fusion and discharge of synaptic vesicles.

Non-exocytotic noradrenaline release i.e. release not mediated by nerve impulses may occur under certain circumstances (Dart *et al.*, 1987) or by indirectly

acting sympathomimetic drugs (e.g. amphetamine), which displace noradrenaline from vesicles.

### **Reflex-mediated Noradrenaline Release**

The reflex-mediated noradrenaline release is caused by the increase in cardiac sympathetic nerve activity. The release of noradrenaline originated from exciting impulses to the heart due to pain and fear, from pressure and volume receptors by a drop in blood pressure and cardiac output, or by reflexes originating directly from the heart *via* afferent nerve fibres (Waldenström *et al.*, 1978; Felder and Thames, 1981).

### **Systemic Release**

The noradrenaline systemic release from heart can be either reflex or due to depolarisation. The contribution of myocardial catecholamines to elevation of catecholamines in the systemic circulation was found to be relatively minor (Esler *et al.*, 1984). The circulating catecholamines mainly originate from peripheral nerve endings (mainly release noradrenaline) and adrenal glands (mainly adrenaline). The circulating catecholamines, within normal range, do not produce arrhythmias in a healthy heart.

Substances brought to nerve terminals *via* the blood stream may reach presynaptic receptors on sympathetic nerve endings in the heart and modulate noradrenaline release. A *milieu* of high  $K^+$  and low pH can affect action potential propagation in neurons, thus presynaptically inhibiting transmitter release (Puig and Kirpekar, 1971; Forfar and Riemersma, 1987; Sanchez-Prieto *et al.*, 1987). This mechanism is of potential significance during myocardial ischaemia when tissue pH falls.

The release caused by reflex-mediated and systemic release requires an intact function of the nerves including their intra-axonal noradrenaline homeostasis, as a prerequisite. The release of noradrenaline induced by increased nerve activity has been shown to be

a energy and calcium dependent process brought about by exocytosis (Douglas and Rubin, 1961).

### **Removal of NA**

Following secretion of noradrenaline by the terminal nerve endings, noradrenaline is removed from the secretory site in three different ways:

- (1). 50 - 80% is removed by active re-uptake (uptake-1) into sympathetic post-ganglionic neurons. This uptake is blocked by tricyclic antidepressant drugs, cocaine, phenoxybenzamine and amphetamine.
- (2). Most of the rest of noradrenaline diffuses away from the nerve endings into surrounding body fluids and tissues, where it is metabolised (uptake-2).
- (3). A minuscule amount of noradrenaline is destroyed by enzymes. Monoamine oxidase is found in nerve endings and catechol-O-methyl transferase is present diffusely in all tissues (Iversen, 1967 and 1975; Lentz, 1983).

### **1.2.2 Noradrenaline Release During Ischaemia**

The elevation of circulating catecholamines can cause an enhanced sensitivity of ischaemic heart to circulating catecholamines. In dog study it has been shown that this results in ventricular arrhythmias (Ungar and Phillips, 1983). The ischaemic heart can achieve its best output at a substantially lower heart rate than that set by its sympathetic control, a result of decreased energy supply which limits the performance of the heart. It is believed that increased sensitivity to circulating catecholamines in ischaemic heart is due to catecholamines reducing efficiency of heart by reducing the duration of heart contraction. This can lead to incomplete transfer of energy to the elastic component of myocardium. Catecholamines accelerate the development of tension and pressure, but they fail to increase peak tension or external work. On the other hand, the metabolic

action of catecholamines, especially the mobilization of free fatty acids, has a toxic effect on the ischaemic heart (Ungar 1979).

There is a progressive release of noradrenaline from the adrenergic nerve terminals of the myocardium after the onset of myocardial ischaemia (Abrahamsson *et al.*, 1984). The overflow of noradrenaline has long been recognised as being of central importance in the development of arrhythmias (Rona, 1985). Depletion of cardiac catecholamine stores by chronic denervation effectively reduced the incidence of ventricular fibrillation induced by experimental coronary ligation (for a review see Schömig *et al.*, 1991).

### Local Release

The local metabolically mediated noradrenaline release from sympathetic nerve endings, in contrast to the systemic and reflex-mediated release, following myocardial ischaemia or anoxia, occurs as a non-exocytotic process. The release is independent of extracellular calcium.

Schömig and colleagues (1984) reported that in the isolated perfused rat heart, when no-flow ischaemia is induced for longer than 15 minutes, there is a progressive noradrenaline overflow during subsequent reperfusion. The results showed that 90% of total noradrenaline overflow is washout from the extracellular space. Dart and Riemersma (1988) showed a correlation between the overflow of noradrenaline and washout of an extracellular space marker. The quantity of noradrenaline overflow during late ischaemia was much greater than that achieved by nerve stimulation (Dart *et al.*, 1984, Schömig *et al.*, 1984). Extracellular noradrenaline concentration may reach 1 - 6  $\mu\text{M}$  range (Schömig *et al.*, 1984), a level which is toxic, even to non-ischaemic myocardium (Rona, 1985; Waldenström *et al.*, 1978). The question is, however, whether such prolonged (20 - 40 minutes) severe ischaemia occurs naturally and whether reperfusion does not exacerbate the effect of ischaemia *per se*. Furthermore, this

ischaemia-induced release of noradrenaline in isolated perfused hearts, where central- and reflex-mediated catecholamine release is excluded, was shown to cease rapidly after reoxygenation with glucose-containing perfusion fluid (Abrahamson *et al.*, 1983; Dart *et al.*, 1987). Noradrenaline release was also observed when isolated hearts were perfused with cyanide. These observations indicate that a major shift in the energy balance is the critical point in the event (Schömig *et al.*, 1987; Dart *et al.*, 1987).

The mechanism of this release is still in debate as it is not possible to measure axoplasmic noradrenaline and  $\text{Na}^+$  concentrations. The experimental evidence indicated that this noradrenaline release can be induced by increase in axoplasmic free noradrenaline concentration, inhibition of MAO activity, and conditions leading to intracellular accumulation of sodium. It was assumed that there is an increase in intra-axonal  $\text{Na}^+$  concentration under ischaemic conditions (Schömig, 1988; Schömig *et al.*, 1988).

Several substances and drugs have been reported to reduce the ischaemia-induced release of noradrenaline by different mechanisms of action. Thus blockers of the uptake-1 carrier e.g. tricyclic antidepressants (desimipramine, *etc.*) have been shown to inhibit noradrenaline release in stop-flow ischaemia. Furthermore, inhibitors of catecholamine biosynthesis and ganglionic blockade were found to be effective.

The metabolic role of this locally-released noradrenaline is to activate anaerobic glycolysis early in ischaemia. However it may also increase calcium influx to the myocyte, contributing to the progression of ischaemic cell injury and death. It is this overflow of noradrenaline along with the ischaemia-induced changes in myocyte function that lead to the development of fatal ventricular fibrillation.

### 1.2.3. Regulation of Noradrenaline Release

#### Metabolic Effect

In the presence of glucose in the perfusate, anoxia itself or uncoupling of oxidative metabolism itself did not cause any increased loss of noradrenaline during ischaemia. In contrast, a marked efflux of noradrenaline was found when the glycolytic pathway was inhibited (Carlsson, 1988).

During ischaemia, the metabolites cannot be removed, which can affect the metabolic changes. i.e. during ischaemia, released noradrenaline can be reuptaken. Acidosis (Haass *et al.*, 1990; Miyazaki and Zipes, 1990) and increased extracellular concentration of  $K^+$  could also inhibit neuronal noradrenaline release during ischaemia (Forfar and Riemersma, 1987; Miyazaki and Zipes, 1990). It is difficult to examine the time course of neuronal noradrenaline release by using stop flow ischaemia. However, the no-flow ischaemia models can provide information on what happens in the centre of the ischaemic area.

#### Anaesthetics

It is well established that pentobarbitone stimulates sympathetic drive. The local anaesthetics have been reported to reduce the ischaemia-induced release of noradrenaline (Schömig, 1988; Polwin *et al.*, 1987; Dart *et al.*, 1983).

### 1.3 CATECHOLAMINE AND GLYCOGEN INTERACTION

#### Chronic Cardiac Denervation and Myocardial Glycogen

The metabolic effects of catecholamines on glucose metabolism include increased glycogenolysis and gluconeogenesis.

It has been suggested that chronic cardiac sympathetic denervation has a protective effect on ischaemia-induced arrhythmias (Gaudeul *et al.*, 1979; Schaal *et al.*, 1969; Ebert *et al.*, 1968). It results in nearly total depletion of myocardial catecholamine stores. Not only does it prevent catecholamine release evoked by central sympathetic activity (this is the case in acute denervation), but obviously there cannot be a local release in response to locally produced metabolic processes. It is therefore more likely that denervation protects by raising the myocardial glycogen content and enhanced provision of glycolytically produced ATP from glycogen (Daugherty *et al.*, 1986).

#### Glycolysis Prevents Noradrenaline Release

Carlsson (1988) reported that with low flow ischaemia ( $0.5 \text{ ml min}^{-1}$ ) there was no noradrenaline release in isolated heart of the rat in the presence of glucose (11 mM), even when glucose was present only during the ischaemic period. Carlsson concluded that maintenance of anaerobic glycolysis is of crucial importance for retention of the noradrenergic transmitter during ischaemic conditions. Dart and colleagues also reported that anoxia itself has no effect on evoked noradrenaline release in the heart when exogenous glucose supply is maintained (Dart *et al.*, 1987; Dart and Riemersma, 1989). One of the difficulties is that the metabolism of glycogen or glucose is measured predominantly in the myocardium. There is no direct information on the effects of glucose or glycogen metabolism in the nerves of the heart.

## **1.4 ANAESTHETICS ON ARRHYTHMIA AND MYOCARDIAL GLYCOGEN**

There have been only a few studies on whether anaesthesia itself influences arrhythmias, and the results are not conclusive. Although ischaemia-induced arrhythmias may not be greatly influenced by anaesthesia (Curtis *et al.*, 1987), reperfusion-induced arrhythmias may be exacerbated by anaesthesia (Bolli *et al.*, 1986). With regard to specific anaesthetics, only halothane has a significant effect, inhibiting arrhythmias in early ischaemia. Halothane has substantial ganglion-blocking activity, unlike pentobarbitone which has no effect (Jang *et al.*, 1983; Kroll and Knight, 1984).

The effects of anaesthetic agents on myocardial glycogen are uncertain, although it is known that anaesthesia can produce metabolic changes including raising plasma glucose and insulin levels which could affect the levels of myocardial glycogen.

Furner and co-workers (1972) studied the effects of sodium pentobarbitone on plasma glucose levels in male Sprague-Dawley rats. The rats were divided into three groups: non-injected, sham-injected and sodium pentobarbitone [25 or 50 mg kg<sup>-1</sup> intraperitoneal injection (ip)] anaesthesia. The results showed that 15 minutes later, the plasma glucose levels significantly increased to 8.3 and 8.9 mM compared with a non-injected control of 7.1 mM. However, the difference was not significant between the sham injected rats and pentobarbitone group until the pentobarbitone dose increased to 50 mg kg<sup>-1</sup>. The plasma glucose then rose to 10.9 mM. It did not show a significant difference after 5, 30 or 60 minutes. They concluded that the increase of plasma glucose is dependent on dose rather than on the duration of anaesthesia.



Arola *et al.* (1981) reported that pentobarbitone ( $40 \text{ mg kg}^{-1} \text{ ip}$ ) significantly increased blood glucose levels at 15 minutes after the anaesthesia, while ether raised the plasma glucose levels both at 15 and 30 minutes. Both anaesthetics raised insulin levels considerably. They conclude that high glucose concentrations in anaesthetised animals seem to be related to decreased glucose utilisation. Ether has also been shown to increase both plasma glucose and insulin levels in humans (Yoshimura *et al.*, 1971).

Aynsley-Green and co-workers found that the effects of anaesthetics are dependent on animal nutritional status. They found that after 30 minutes of anaesthesia in 48 hour starved Wistar rats, both ether and halothane treated rats had a significant rise in fasting blood sugar. Ether, but not halothane, also raised plasma insulin. In the pentobarbitone ( $30 \text{ mg kg}^{-1}$ , intravenously) group, there was no change in fasting blood glucose or plasma insulin at 5 minutes after the induction of anaesthesia. In the fed group, halothane caused a highly significant 50% fall in plasma insulin 30 minutes after induction of anaesthesia which was accompanied by a small rise in blood glucose. A similar change was also found 5 minutes after pentobarbitone induction (Aynsley-Green *et al.*, 1973).

Animals are usually anaesthetised for studies of myocardial glycogen metabolism. The metabolic changes by anaesthetics mentioned above could influence the metabolism of the heart. However, few studies were actually designed to examine the effect of anaesthetics on myocardial glycogen levels and its metabolism.

## 1.5 WORKING PROJECT

The mechanisms responsible for changing from a reversible to an irreversible state of damage transition are still not known with certainty. Enhanced myocardial glycogen has been shown to be able, under certain conditions, to protect the heart from fatal arrhythmia, but the beneficial effects remain controversial. The precise conditions under which the protective effects of glycogen might occur and the electrophysiological mechanisms of such effects are still not fully understood. To elucidate these conditions might lead to a better means of prevention of sudden arrhythmic death in humans.

Two of the main mechanisms of myocyte cell death in severe ischaemia are increased circulating catecholamines and inadequate supply of glycolytically produced ATP (Opie 1993). There is no evidence that myocardial glycogen affects NA release during ischaemia. To address this issue, a study could provide an explanation of the mechanisms of the protective effect of myocardial glycogen on VF. The evidence of the protective effects of glucose on ischaemic NA release mentioned earlier has led to the following hypothesis: Increased myocardial glycogen pre-ischaemia may decrease or delay ischaemic induced NA release. Attention will be focused on the effects of anaerobic glycolysis on myocardium. In particular, the effect of glycogen on anoxia-induced catecholamine release. The possible mechanisms will be discussed (Chapter 3).

The effects of anaesthetics on myocardial glycogen are unknown. As all rats would be anaesthetised before operation (expert stunned control), the relationship between anaesthetics or anaesthetic conditions and myocardial glycogen levels is also emphasised (Chapter 4).

Fasting is one of the methods of increasing myocardial glycogen. Because of uncertainties regarding the influence of fasting on ischaemia-induced VF *in vitro*, this has been studied (Chapter 5).

## **CHAPTER 2**

### **Methodology**

## **2.1 EXPERIMENTAL MODEL AND ANIMAL HOUSING CONDITIONS**

Male Sprague-Dawley rats of 300 - 500 g body weight (Bantin and Kingman Ltd, North Humberside, UK) were used throughout. All animals were treated humanely and in compliance with the guidelines from the Faculty of Medicine Animal Area (FMAA) Users' Committee of the University of Edinburgh and the Home Office, UK.

The rats were housed at a constant temperature of 21 - 22 °C, with a cycle of 12 hours artificial light and 12 hours dark. Each cage of 50 x 36 x 22 cm contained four rats. The animals had free access to food (Rat and Mouse Standard Diet, Bantin and Kingman, UK) and tap water. For some experiments, rats were fasted with free access to water for 24 or 48 hours.

All experiments were carried out at least three days after receipt of the rats by the FMAA.

## **2.2 ANAESTHESIA**

The following anaesthetics were used: sodium pentobarbitone (May and Baker Ltd, Dagenham, UK) intraperitoneal (ip.) injection 60 mg kg<sup>-1</sup> body weight; halothane 4 - 5% in 95%O<sub>2</sub> : 5% CO<sub>2</sub> (2.5 L min<sup>-1</sup>, ICI Pharmaceuticals, Macclesfield, UK); peroxide-free diethylether (Merck Ltd, Glasgow, UK).

During anaesthesia, the rat was maintained at room temperature. Rectal temperature was not measured.

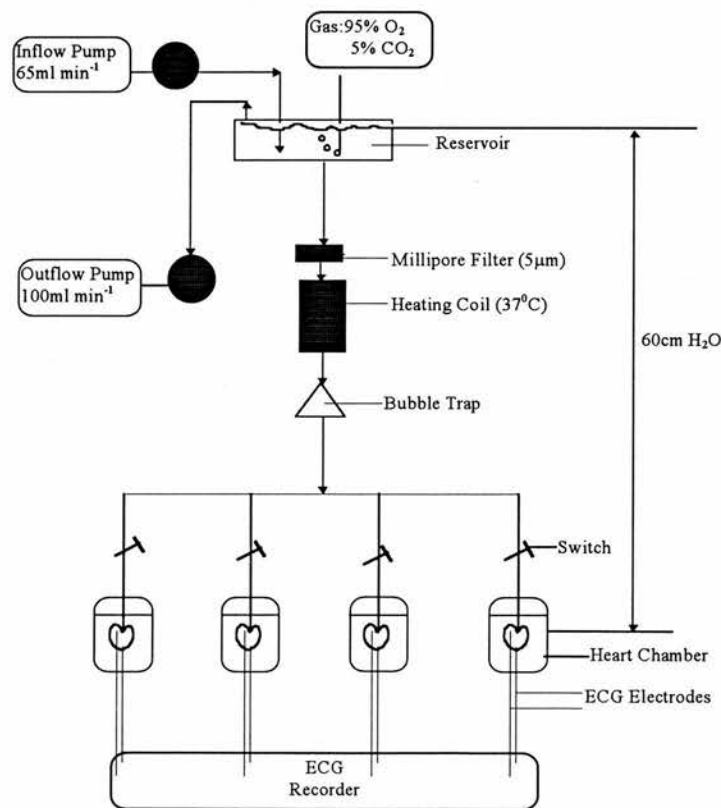
2.3 LANGENDORFF PERFUSION SYSTEM

A technique developed by Oscar Langendorff more than 100 years ago was used to perfuse the isolated rat hearts. The principle of the technique is that the heart is subjected to an aortic retrograde perfusion (Taegtmeyer, 1995).

The coronary flow was controlled by the following settings:

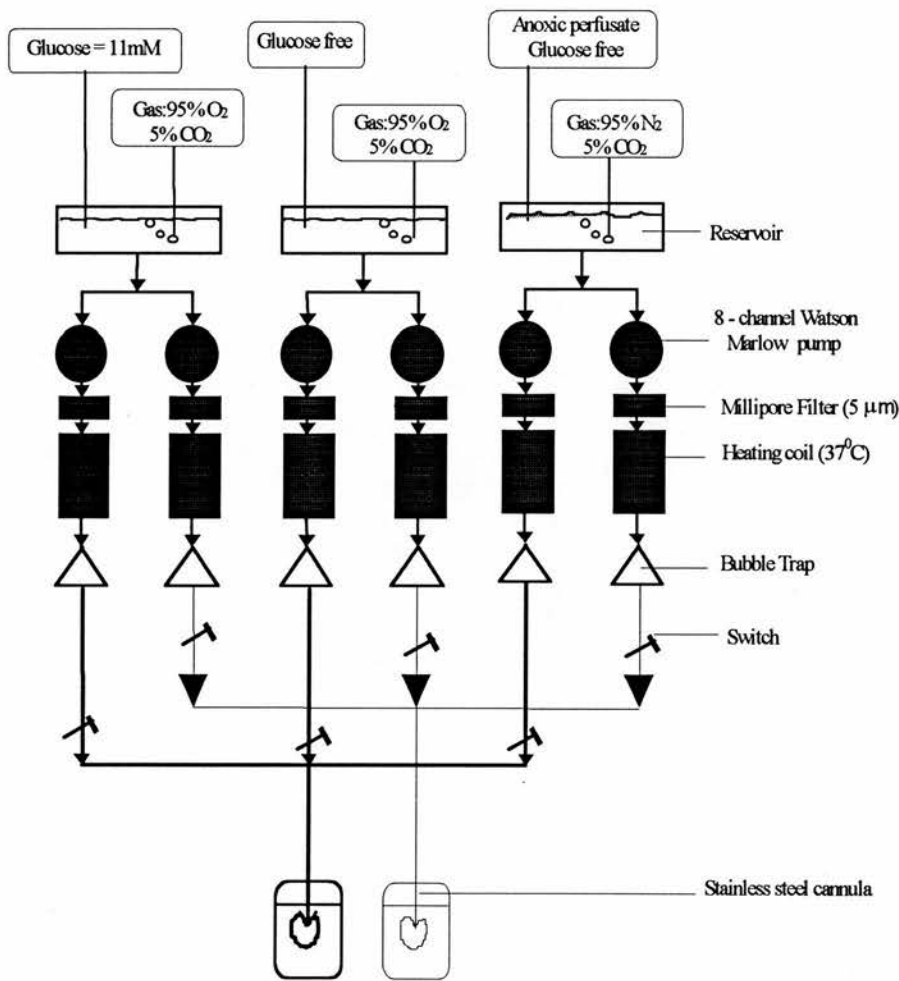
- 1). A constant perfusion pressure of 60 cm H<sub>2</sub>O. Four hearts were perfused simultaneously (Fig. 2.1).

**Fig. 2.1** Schematic illustration of the apparatus for pressure controlled Langendorff Perfusion system with constant perfusion pressure (60 cm H<sub>2</sub>O). The level of perfusate in the reservoir was constant, this was controlled by 2 pumps. The temperature of the perfusate was kept constant at 37 °C by use of thermostat with water-jacketed perfusion lines. The heart chamber also provided a constant temperature (37 °C) for the heart even when perfusate flow was stopped. The electrocardiogram (ECG) was obtained using 2 silver electrodes, which were attached to the anterior wall of the left ventricle near the apex and the right atrium. The ECG was recorded at a paper speed of 2.5 cm second<sup>-1</sup>. Four hearts were studied simultaneously.



2). A constant perfusion flow rate system was also used in some experiments with the aid of a Watson Marlow multichannel peristaltic pump (202U) [9.9 (0.1) ml min<sup>-1</sup>, n = 16; or 52 (0.4) ml g<sup>-1</sup> dry wt min<sup>-1</sup>]. Two hearts were mounted in chambers consecutively. Three reservoirs were included in order to modify perfusion composition. This system was used in anoxia studies in order to obtain a constant outflow to determine noradrenaline overflow (Fig. 2.2).

**Fig. 2.2** Flow controlled Langendorff perfusion system  
Two hearts were studied simultaneously.



Glycolytic flux in the myocardium is sensitive to the adequacy of the coronary flow rate (Neely *et al.*, 1975). The flow was therefore measured in all the experiments.

To make the data comparable with previous work in our department (Sargent, 1990), the perfusion pressure of 60 cm H<sub>2</sub>O was used. This is similar to the pressure used by Opie *et al.* (1980), which was 65 cm H<sub>2</sub>O. The pressure of non-ligated coronary arteries was unchanged after ischaemia which was equivalent to the perfusion pressure.

The perfusion pressure was not measured in the flow-constant perfusion setting used in Chapter 3, as the aim of the experiments was not to examine the effects of insulin and glycogen on coronary pressure changes, but their effects on NA overflow. In these experiments global anoxia was induced with maintained flow.

## **2.4 OPERATION PROCEDURE**

After the rat was anaesthetised (the rat became unresponsive to pinching the toe), the femoral vein was exposed. 0.25 units g<sup>-1</sup> body weight heparin (Leo Laboratories Limited, Bucks.) was administered into the vein at least 30 seconds before thoracotomy to allow adequate distribution throughout the blood pool. When a blood sample was required, the abdomen was opened and 1.5 - 2 ml blood was collected using a heparinised plastic syringe from the abdominal aorta. The procedure took less than 15 seconds.

The chest was then opened. The heart was removed and transferred to ice cold oxygenated perfusate within 20 seconds of thoracotomy. The heart was rinsed, and was taken out of the ice-cold perfusate as soon as it stopped beating. The heart was then

mounted on the perfusion system by cannulating the aorta using a stainless steel cannula (outer diameter 2.2 mm).

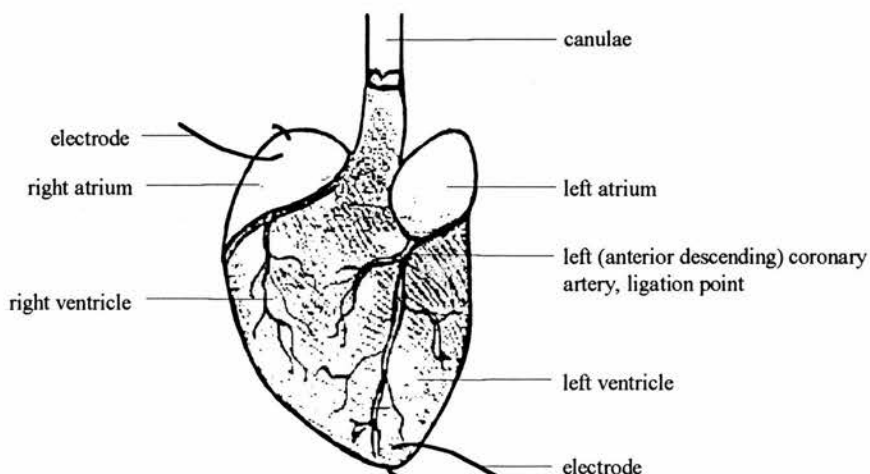
The perfusate was allowed to flow freely through the cannula whereupon the heart restarted beating. The heart was held in place using a 2/0 Mersilk thread (purchased from Ethicon Ltd., UK). Experiments consisting of 4 hearts were suspended within 10 minutes in total, those of 2 rats within 5 minutes. A 10 minute equilibrium perfusion was always allowed before each experiment.

## 2.5 PLACEMENT OF LIGATURE AND INDUCING ISCHAEMIA

In the rat heart, the ascending aorta, gives rise to only two branches, the right and left coronary arteries, which leave the aorta close to its origin and run downward over the heart supplying its walls (Greene, 1963).

Acute regional myocardial ischaemia was induced by ligation of the left (anterior descending) coronary artery (LAD). A Mersilk 4/0 16 mm suture (Ethicon Ltd., UK) was placed around the LAD (Fig. 2.3). The two ends of each suture were left free until ligation. The hearts were then allowed to recover for 7 - 10 minutes.

**Fig. 2.3** Ligation point of the coronary artery in the rat heart.





To identify ischaemic and non-ischaemic area, Evans blue (1%, 0.5 ml) was injected through a side arm of the aortic cannula just before freeze clamping. The results did not show a significant difference in ischaemic and non-ischaemic tissue of both fed and fasted rat hearts (Table 2.1). There were also no significant difference in ischaemic proportions between VF and non-VF control hearts (Table 2.2). Evans blue at the above concentration did not interfere with the spectrophotometric assays.

**Table 2.1** Ischaemic tissue weight of control and VF hearts of fed and fasted rats.

|              | Control     |             | VF          |             |
|--------------|-------------|-------------|-------------|-------------|
|              | heart wt.   | Isc. wt.    | heart wt.   | Isc. wt.    |
| Fed (n=10)   | 0.229(0.01) | 0.106(0.01) | 0.220(0.01) | 0.096(0.01) |
| Fasted (n=7) | 0.210(0.01) | 0.09(0.01)  | 0.216(0.01) | 0.1(0.01)   |

The results are expressed as mean (sem) for the number of hearts given (n). Evens Blue was used to identify ischaemic tissue. Isc. wt: ischaemic (tissue) weight.

**Table 2.2** Ischaemic proportions (ischaemic tissue weight / whole heart weight x %) of control and VF hearts of fed and fasted rats.

| Isc. area %  | Control | VF    | t-test (p) |
|--------------|---------|-------|------------|
| Fed (n=10)   | 46(4)   | 43(3) | 0.6        |
| Fasted (n=7) | 43(2)   | 46(4) | 0.5        |

The hearts are the same as in Table 2.1. The results are expressed as mean (sem) for the number of hearts given (n).

## 2.6 THE PERFUSION MEDIUM

All hearts were perfused with a non-recirculating modified Ringer-Locke solution with 3 mM K<sup>+</sup>. The perfusate was freshly made up using double distilled water. When Ca<sup>++</sup> was

required, perfusate of 5 litre was gassed with CO<sub>2</sub> for 15 minutes before Ca<sup>++</sup> (50 ml of 3.68% CaCl<sub>2</sub>.2H<sub>2</sub>O) was added to prevent a precipitate of Ca (OH)<sub>2</sub> being formed.

Calcium free perfusate was prepared by omitting calcium from the perfusate. 0.1 mM EGTA (Lew *et al.*, 1985) was added to the perfusate to chelate any residual calcium.

All chemicals were analytical grade and supplied by the Sigma or BDH Chemical Companies, UK. The ionic concentrations are shown in Table 2.3.

**Table 2.3.** Perfusate ionic concentrations

| Ion                            | Concentration<br>(mM) |
|--------------------------------|-----------------------|
| K <sup>+</sup>                 | 3.0 or 4.0            |
| Na <sup>+</sup>                | 141                   |
| Cl <sup>-</sup>                | 126                   |
| Ca <sup>++</sup>               | 2.5 or 0              |
| Mg <sup>++</sup>               | 1.0                   |
| PO <sub>4</sub> <sup>---</sup> | 0.4                   |
| HCO <sub>3</sub> <sup>-</sup>  | 25                    |

The perfusate was maintained at 37 °C, gassed with 95% O<sub>2</sub> : 5% CO<sub>2</sub>. The perfusate was passed through a mixed cellulose acetate and nitrate millipore filter (5 µm) before it entered the heart. Samples of the perfusate were collected from the reservoir anaerobically just before and after perfusing the heart. The pH, pO<sub>2</sub>, pCO<sub>2</sub> of the perfusate (Table 2.4) were measured by a pH/Blood Gas Analyser (Instrumentation Laboratory System 1302, Warrington, UK).

**Table 2.4** Typical pH, pO<sub>2</sub> and pCO<sub>2</sub> in the perfusate.

|                  |                         |
|------------------|-------------------------|
| pH               | 7.38 (0.007)            |
| pO <sub>2</sub>  | 543 (10) mmHg (Control) |
|                  | 0 or 1 mmHg (Anoxia) *  |
| pCO <sub>2</sub> | 37 (1) mmHg             |

\*: gased with 95% N<sub>2</sub> : 5% CO<sub>2</sub>.

The data are from 12 consecutive experiments and expressed as mean (sem).

## ANOXIA

Anoxia (pO<sub>2</sub> < or = 1 mmHg) was induced by gassing the perfusion fluid with 95% N<sub>2</sub> : 5% CO<sub>2</sub> and was confirmed by pO<sub>2</sub> measurement. The addition of the reducing agent, sodium dithionite with a final concentration of 0.5 mM was used to remove remaining traces of oxygen (Dart *et al.*, 1987).

## POTASSIUM

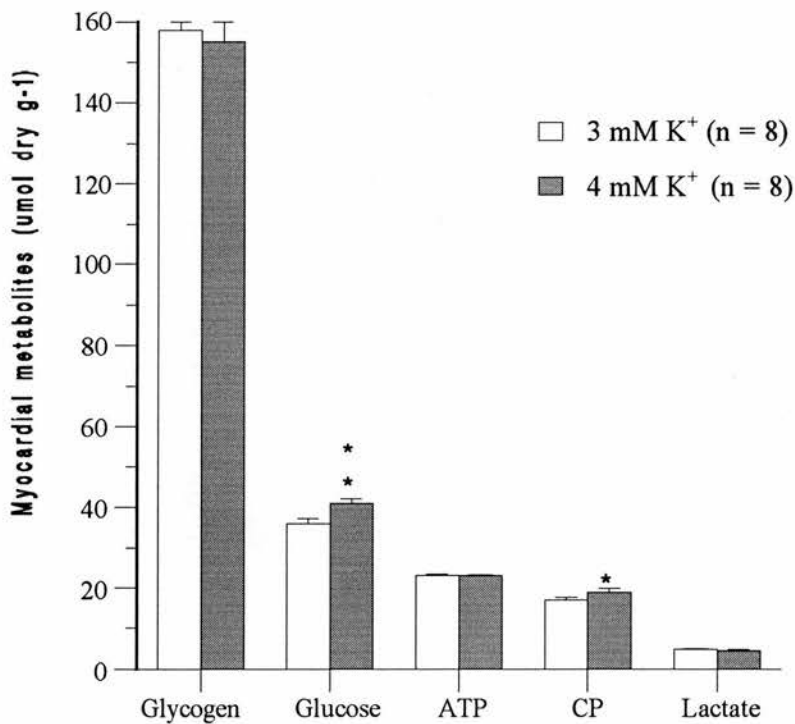
The standard [K<sup>+</sup>] level (4 mM) in plasma was used in the experiments, except in the arrhythmia study. It is known that in the rat hearts, ischaemia rarely induces arrhythmia when 4 mM [K<sup>+</sup>] is applied. It is well known that hypokalemia increases the ventricular vulnerability to arrhythmias both clinically and experimentally (Nordrehaug and Vander-Lippe, 1983; Curtis and Hearse, 1989). The 3 mM [K<sup>+</sup>] level was used in order to induce a higher incidence of arrhythmia and also to make the data comparable with other arrhythmia studies in our department (Sargent, 1990).

The effects of different potassium levels (K<sup>+</sup> 3 or 4 mM) on myocardial glycogen and metabolites levels of perfused hearts have been studied. The results showed that different potassium concentrations did not affect myocardial glycogen concentrations.

However, hearts perfused with 4 mM potassium had higher myocardial free glucose and CP levels (Fig. 2.4).

**Fig. 2.4** Myocardial glycogen and metabolites in isolated rat hearts.

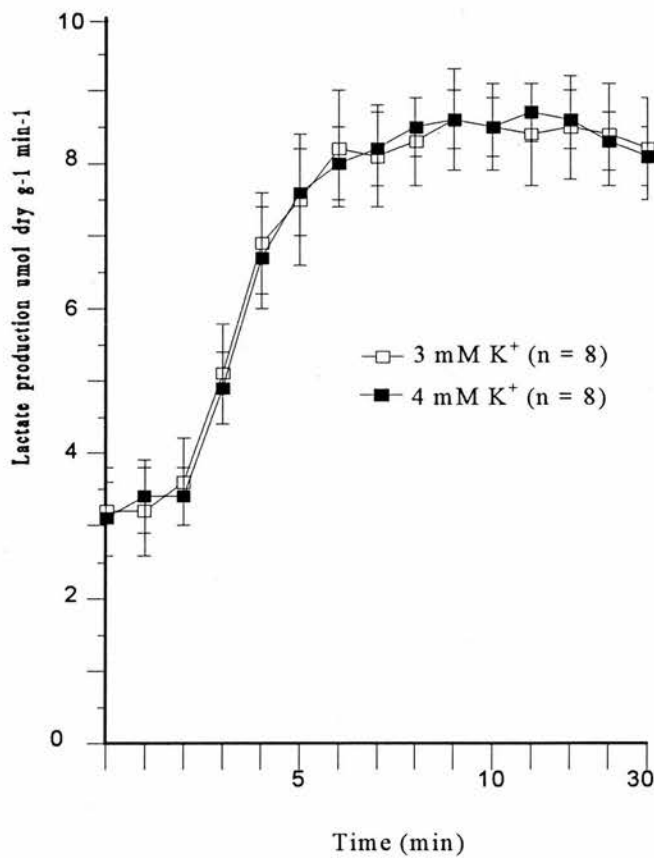
The perfusate contained 11 mM glucose and 2000  $\mu\text{U ml}^{-1}$  insulin. The experimental hearts were perfused with 4 mM  $\text{K}^+$ . The perfusion flow was constant. \*:  $p < 0.05$ , \*\*:  $p < 0.01$  vs. 3 mM  $\text{K}^+$  control, t - test. Each point represents the mean with vertical lines indicating s.e.m..



The tissue lactate level was unchanged. There was no significant difference in myocardial lactate production (30 minutes area under curve) between the perfusate containing 3 mM  $\text{K}^+$  [233 (18)  $\mu\text{mol g}^{-1}$  dry wt 30  $\text{min}^{-1}$ ] and 4 mM  $\text{K}^+$  [235 (11)  $\mu\text{mol g}^{-1}$  dry wt 30  $\text{min}^{-1}$ ] perfused hearts (Fig. 2.5).

**Fig. 2.5** Lactate production in isolated rat hearts.

Hearts are the same as Fig. 2.4. The experimental hearts were perfused with 4 mM K<sup>+</sup>. The perfusate contained insulin throughout. The lactate in the effluent rose with time ( $p < 0.05$ ) but not if K<sup>+</sup> levels were altered (two way analysis of variance). Each point represents the mean with vertical lines indicating s.e.m..



**GLUCOSE**

The perfusate contained 5.5 mM glucose unless otherwise indicated. A comparison was made to determine myocardial metabolites when using different glucose levels in the perfusate. Increasing glucose from 5.5 to 11 mM did not affect myocardial metabolites (Table 2.5). When glucose in the perfusate was kept constant (11 mM), hearts perfused

with insulin had higher myocardial glycogen, CP levels and tissue lactate production, but not ATP levels.

**Table 2.5** Myocardial metabolites in isolated rat hearts.

| Myocardial metabolites<br>( $\mu\text{mol g}^{-1}$ dry wt) | Glucose 11 mM<br>(n = 8) | Glucose 11 mM<br>+ Insulin 2000 $\mu\text{U l}^{-1}$ (n = 7) | Glucose 5.5 mM<br>(n = 7) |
|--|--------------------------|--|---------------------------|
| Glycogen   | 81 (5)                   | 157 (7)***   | 87 (5)                    |
| Free Glucose   | 28 (1)                   | 37 (2) **  | 29 (1)                    |
| ATP  | 21 (1)                   | 22 (1)   | 21 (0.4)                  |
| CP   | 13 (1)                   | 18 (1) ***   | 14 (1)                    |
| Lactate  | 1.9 (0.4)                | 3.7 (0.3)**  | 2.4 (0.3)                 |

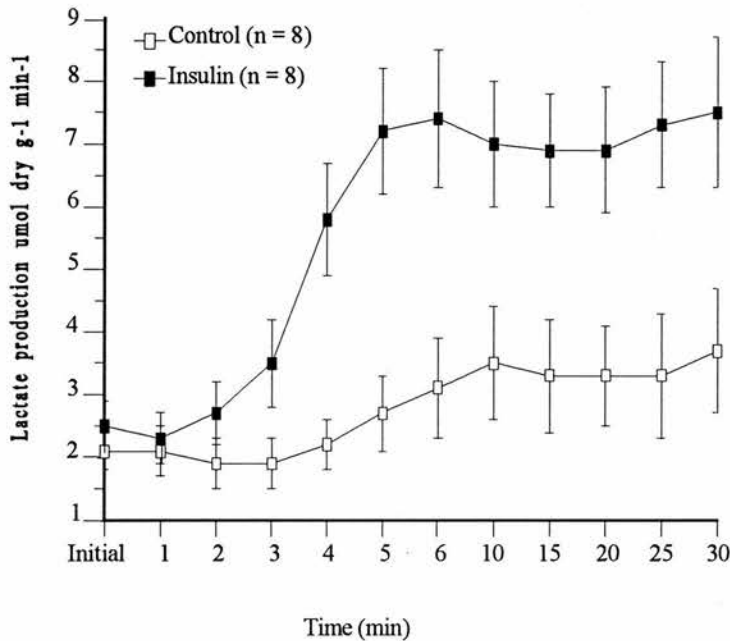
The results of myocardial metabolites are expressed as mean (sem) of hearts shown (n). The experimental hearts were perfused with insulin or 5.5 mM glucose. Perfusion with constant flow of 10 ml  $\text{min}^{-1}$  with 4 mM  $\text{K}^{+}$  for 30 minutes. \*\*:  $p < 0.01$ , \*\*\*:  $p < 0.001$  (t - test, vs. control).

## INSULIN

Human insulin (Velosulin, 100 units  $\text{ml}^{-1}$ , Novo Nordisk A/s, Denmark) was infused using a Harvard infusion pump and a 10 ml plastic syringe to prevent absorption to glassware. Final insulin concentration in the perfusate was approximately 2000  $\mu\text{U ml}^{-1}$ . The coronary production of lactate was affected by the duration of perfusion ( $p < 0.05$ ) and by insulin ( $p < 0.01$ ), there was no interaction between insulin and the duration of perfusion ( $p > 0.05$ , two-way analysis of variance). The total lactate production [area under the curve; mean (sem)  $\mu\text{mol l}^{-1} \text{g}^{-1}$  dry wt 30  $\text{min}^{-1}$ ] was significantly higher ( $p = 0.01$ , t - test.) in insulin perfused hearts [197 (26)] than control [93 (23)]. The outflow of lactate increased significantly after 3 minutes of perfusion with insulin (Fig. 2.6). The same pattern can be seen in Fig. 2.5.

**Fig. 2.6** Coronary lactate production in isolated hearts.

The experimental hearts were perfused with insulin. Perfusate contained 11 mM glucose and 4 mM  $K^+$ . Each point represents the mean with vertical lines indicating s.e.m..



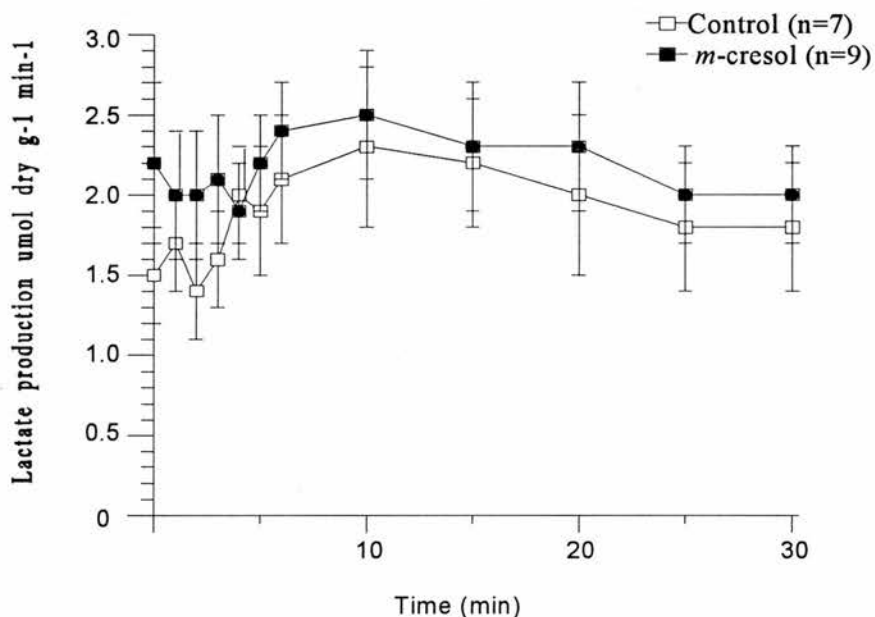
The insulin used (human velosulin) contained 3 mg ml<sup>-1</sup> *m*-cresol which is a preservative to prevent contamination by microbes. An investigation was carried out to examine whether *m*-cresol itself affects the level of myocardial glycogen and metabolites. The *m*-cresol at a final concentration of 0.06 mg l<sup>-1</sup> (0.56 mM) was in the perfusate. This *m*-cresol concentration is equivalent to its level in a perfusate containing 2000 μU ml<sup>-1</sup> insulin. Hearts were perfused for 30 minutes with constant flow of 10 ml minute<sup>-1</sup> (glucose = 11 mM,  $K^+$  = 4 mM). The results did not show that myocardial metabolites (μmol g<sup>-1</sup> dry wt) were affected [*m*-cresol free control (n = 7) vs. *m*-cresol (n = 9); glycogen: 90 (5) vs. 92 (8); Free glucose: 29 (2) vs. 29 (1); ATP: 21 (0.4) vs. 20 (1); CP: 8 (1) vs. 9 (1); tissue lactate: 4 (0.3) vs. 5 (1),  $p > 0.5$ , *t*-tests].

Lactate production [mean (sem) μmol g<sup>-1</sup> dry wt min<sup>-1</sup>] was not affected by either perfusion duration or *m*-cresol (two way analysis of variance) (Fig. 2.7). Total lactate

production (area under curve,  $\mu\text{mol g}^{-1} \text{ dry wt } 30 \text{ min}^{-1}$ ) was 58 (12) in control and 65 (10) in *m*-cresol perfused hearts.

**Fig. 2.7** Lactate production in isolated perfused rat hearts.

The experimental hearts were perfused with *m*-cresol. Each point represents the mean with vertical lines indicating s.e.m..



## 2.7 ELECTROCARDIOGRAPHIC MONITORING

The electrocardiogram (ECG) was obtained using 2 silver electrodes, one attached to the anterior wall of the left ventricle near the apex and the other to the right atrium (Fig. 2.3). It recorded in the experiments of Chapter 5 only. The ECG was monitored continuously from a 4-channel oscilloscope and recorded at a paper speed of  $2.5 \text{ cm second}^{-1}$  (Gould TA 2000 recorder, USA).



## 2.8 DEFINITION OF VT AND VF

Ventricular tachycardia (VT) was defined as a run of four or more ventricular premature beats (VPB) irrespective of the prevailing sinus rate. Ventricular fibrillation (VF) was defined as a signal for which individual QRS deflections could no longer be identified and for which the rate could no longer be measured. The recommendations of the Lambeth Convention were used for the analysis of those arrhythmias (Walker *et al.*, 1988).

In the studies of ventricular arrhythmias, all the hearts with VF following VT except one heart of fed rat (Table 2.6).

**Table 2.6** The onset (minutes) and duration (minutes) of VT and VF after LAD ligation in fed and fasted rat hearts.

|             |                      | VT onset  | VT duration | VF onset   | VF duration |
|-------------|----------------------|-----------|-------------|------------|-------------|
| Fed rats    | Control hearts (n=9) | 8.0 (0.4) | 0.6 (0.1)   |            |             |
|             | VF hearts (n=10)     | 7.3 (0.2) | 0.5 (0.1)   | 10.4 (0.7) | 0.3 (0.05)  |
| Fasted rats | Control hearts (n=6) | 8.6 (0.6) | 1.1 (0.3)   |            |             |
|             | VF hearts (n=7)      | 7.8 (0.5) | 1.2 (0.3)   | 12.1 (1.1) | 0.37 (0.03) |

Hearts are the same as those in Table 5.4. There are no significant differences between control and VF hearts. All the hearts with fibrillation following VT except one heart of fed rat. The results are expressed as mean (sem) for the number of hearts given (n).

## 2.9 SAMPLE COLLECTION, PREPARATION AND STORAGE

The coronary effluent was collected for 5 seconds to measure noradrenaline levels or for 30 seconds to estimate lactate and glucose. Lactate was measured either within a 60 minute period after collection or stored at - 40 °C in a freezer before assay.

At the end of each experiment, to stop chemical or enzymatic transformation of the tissue, the heart or liver was rapidly frozen using a Wollenberger clamp pre-cooled to

the temperature of liquid nitrogen ( $-196^{\circ}\text{C}$ ). The temperature of a heart (guinea-pig heart study) can be reduced to  $-10^{\circ}\text{C}$  within 3 seconds to achieve an effective cessation of chemical reactions. The freeze clamped tissue was immediately immersed in liquid nitrogen.

The tissue was then freeze dried (Modulyo S/N1810 freeze drier; Edwards, Merck Ltd., U.K.) for 48 hours. The heart atria were removed, the weight of ventricles was recorded.

The tissue was then pulverised by a dismembrator using a stainless steel ball (Braun Mikrodismembrator II, McKay and Lynn, Edinburgh, U.K.). Each powdered sample (ca. 50 mg) was weighed and mixed with 3 ml 0.6 M perchloric acid in a 10 ml polypropylene tube. A 200  $\mu\text{l}$  sample of the homogeneous suspension was taken for glycogen determination and was placed on ice. 40  $\mu\text{l}$  of a matched potassium carbonate solution (approx. 0.3 M) was then added to neutralise the perchloric acid to  $\text{pH} = 8$ . Once mixed, the tube was stoppered and stored at  $-40^{\circ}\text{C}$  until analysis.

The remaining suspension was placed in a refrigerated centrifuge (Centra-7R, International Equipment Company, USA) at  $4^{\circ}\text{C}$ , 2000 x g for 20 minutes. 2 ml supernatant was combined with 400  $\mu\text{l}$  of 0.3 M potassium carbonate and kept on ice for 30 minutes. The sample was re-centrifuged as above. The supernatant was taken for the analyses of ATP, CP, free glucose and lactate.

## **2.10 BIOCHEMICAL ANALYSES**

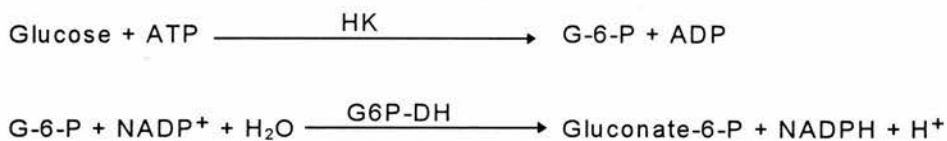
All biochemical analyses were carried out using a centrifugal analyser (Cobas Bio centrifugal analyser: Roche Diagnostics, Welwyn Garden City, Herts, UK). All samples from experimental and control groups were assayed together in duplicate.

Heart weight was expressed as dry weight (g dry wt), which is 22.4 - 24.3% of wet weight (Adrouny and Russell, 1956). The heart weight throughout the studies (i.e. the weight of ventricles) was recorded. The dry weight of atria was 5(0.1)% of the whole heart. The weight of whole heart: mean (sem) = 0.25 (0.003) g dry weight, the weight of ventricles = 0.236 (0.002) g dry wt. The body weight of the rat was on average 342 (3) g, n = 39.

The tissue glycogen, free glucose, ATP, CP and lactate values were expressed as  $\mu\text{mol g}^{-1}$  dry wt; NA overflow as  $\text{pmol g}^{-1}$  dry wt  $\text{min}^{-1}$ ; and the lactate production in  $\mu\text{mol g}^{-1}$  dry wt  $\text{min}^{-1}$ .

### 2.10.1 GLUCOSE

Glucose was analysed using a Glucoquant Test Combination kit (Boehringer Mannheim GmbH Diagnostica). The glucose standard used for the assay was Preciset-D-glucose (0.505 mM, Boehringer Mannheim GmbH Diagnostica). Glucose was phosphorylated to glucose-6-phosphate (G-6-P) using hexokinase (HK) in the presence of  $\text{Mg}^{++}$  and ATP. The G-6-P thus generated was oxidised by G-6-P dehydrogenase (G6P-DH) in the presence of  $\text{NADP}^+$  (nicotinamide adenine dinucleotide phosphate) leading to NADPH [nicotinamide adenine dinucleotide phosphate (reduced)]. The concentration of the NADPH could be measured by the change of absorbance at 340 nm, at 37 °C. The absorbance was proportional to the amount of glucose. The reactions are summarised as follows:



A glucose plasma pool was used for quality control. The coefficient of variation (CV) of this method was 3.9% [between assay variability, mean (sem) = 197 (2)  $\mu\text{M}$ , n = 13]. The

within assay variability was determined by 24 repeated analyses of one sample. The within assay CV was 0.59%.

### 2.10.2 GLYCOGEN

Estimation of myocardial glycogen concentration was based on the enzyme-linked spectrophotometric assay of Keppler and Decker (1974) with modifications to suit the Cobas Bio. The method is based on quantitative glycogen hydrolysis followed by measurement of glucose (section 2.10.1).

#### Stage 1: Conversion of glycogen to glucose



Samples and standards were hydrolysed using 1 mg ml<sup>-1</sup> amyloglucosidase (from *Aspergillus niger*, Boehringer Mannheim 102 857) in 0.2 M acetate buffer (pH 4.8) for two hours at 40 °C. 0.5 ml, 0.6 M perchloric acid was added and the samples were kept ice cold for 15 minutes. Potassium carbonate (0.1 ml, 0.3 M) was then added. The samples were then kept on ice for a further 30 minutes. KClO<sub>4</sub> was precipitated by centrifugation at 4 °C, 1200 x g for 15 minutes. The supernatant was then transferred to Cobas Bio cups for glucose analysis.

#### Stage 2: Glucose Analysis

Glucose was then analysed in accordance with procedures given above (Section 2.10.1).

Standards: A range of standards from 0.1 to 0.5 g l<sup>-1</sup> was prepared by using commercially available glycogen (BDH Chemicals; from mammalian liver). Paired 200 µl aliquots of each standard were dispensed into 10 ml plastic tubes. Duplicated samples of

the neutralised perchloric acid served as blanks. Standards and samples received identical treatment during the analysis.

The CV of the method (between assay variability) was 4% [mean (sem) = 201 (2)  $\mu$ M, n = 12]. The within batch CV was determined by 24 times repeated analysis of a mixed glycogen sample from one experiment in one rotor. It was 0.47%. Glycogen recovery was 86(2)% (n = 8). It was determined by addition of pure glycogen (used for standards, see above) to samples of 8 different experiments.

The myocardial glycogen levels, from the method described above, included tissue extracellular free glucose. This could affect the apparent tissue glycogen results when the perfusate contained different levels of glucose. The following study was therefore performed. The isolated hearts were perfused with 5.5 mM glucose for an equilibrium perfusion of 10 minutes as control. The experimental hearts were perfused for 30 minutes (after equilibrium perfusion) with 15 mM glucose. The results (Table 2.7) showed that after subtraction of free glucose, glycogen levels did not differ between 15 mM glucose perfused hearts and control. The myocardial glycogen results were therefore, in all experiments, presented as 'pure' glycogen, which did not contain tissue free glucose.

**Table 2.7** Myocardial glycogen and free glucose levels ( $\mu$ mol g<sup>-1</sup> dry wt) in the isolated perfused heart.

| Perfusate glucose | Glycogen+free glucose | Free glucose | Glycogen  |
|-------------------|-----------------------|--------------|-----------|
| 5.5 mM (n = 7)    | 120 (5.5)             | 8.9 (0.4)    | 111 (5.6) |
| 15 mM (n = 10)    | 141 (5.7)             | 29.2 (0.7)   | 112 (6.1) |
| t - test          | p = 0.02              | p < 0.0001   | p = 0.95  |

The results are expressed as mean (sem) for the number of hearts shown (n). The experimental hearts were perfused with 15 mM glucose.

### 2.10.3 ATP AND CP

The concentrations of tissue ATP and CP were determined by using a coupled enzymatic and ultra violet (UV) method (Lamprecht and Trautschold 1974) adapted for use on the Cobas Bio. The principle of the method is as follows:



The first 2 reactions permit the determination of sample ATP by measuring the increase in absorbance at 340 nm due to the formation of phosphogluconate and NADPH. When this reaction came to completion, and after all ATP of the sample had been consumed, the level of CP was determined by the addition of creatine kinase (CK).

Standards: The method was calibrated using ATP and CP dissolved in neutralised perchloric acid. The final concentrations were 750  $\mu\text{M}$  and 1125  $\mu\text{M}$  respectively.

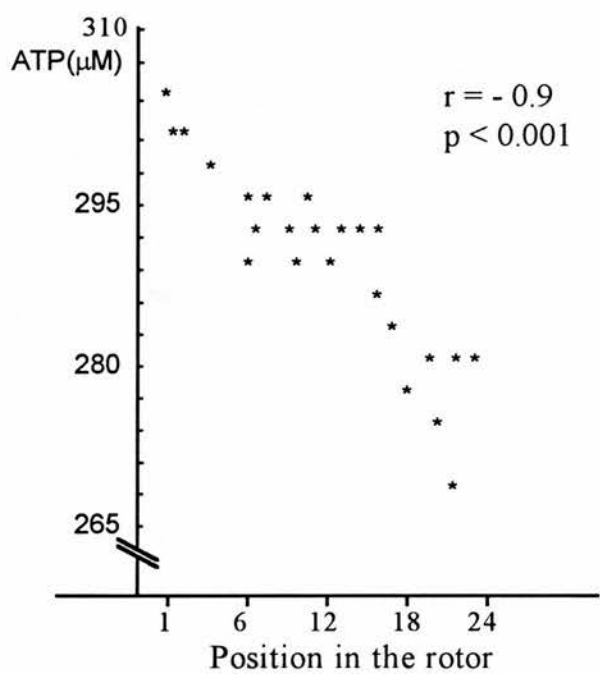
The use of a 'blanking mode' on the Cobas Bio enabled consecutive measurement of ATP and CP, i.e. the final optical density reading for the ATP assay served as the blank for that of CP.

After transfer of samples and reagents, a short incubation period was allowed before starting the reaction. The start reagent for the ATP assay was HK and for the CP was CK. The final concentrations of HK in cuvette was 70 U.  $\text{ml}^{-1}$  and of CK 4800 U.  $\text{ml}^{-1}$ .

The pipetting of all the samples ( $n=24$ ) and reagents into the thermostatted Cobas Bio rotor took a few minutes. It is therefore possible that at the start of the reaction there was a difference in sample temperatures between various cuvettes, the samples pipetted at

the beginning being closer to the set temperature than the last samples of an analytical run. The repeated analysis of the same ATP sample (305  $\mu$ M) shows slightly lower results for the samples pipetted last (Fig. 2.8). The correlation coefficient ( $r$ ) was 0.9 ( $p < 0.001$ ). This did not occur when measuring tissue glycogen ( $r = - 0.02$ ); free glucose ( $r = - 0.2$ ), lactate ( $r = 0.2$ ) and CP ( $r = - 0.1$ ) when samples were incubated for the same periods.

**Fig. 2.8** Myocardial ATP levels from one sample in relation to position on the rotor  
One sample containing ATP was distributed over 24 sample cups of one rotor of Cobas Bio.



The auxiliary absorbance reading (an extra reading the machine makes) was recorded before HK was added. The optical density was higher according to the position of the sample cup on the rotor ( $r = 0.7$ ,  $p < 0.001$ ). The position did not affect the final absorbance reading against time or the initial readings. When the incubation period was increased from 10 to 80 seconds, the increased incubation time eliminated these

positional effects and there was no longer a relation between rotor position and absorbance ( $r = 0.15$ ,  $p > 0.1$ ).

A significant relationship remained between apparent ATP and sample position ( $r = -0.61$ ,  $p < 0.01$ ; not shown). This is most likely due to the light sensitivity of NADPH. After the corrections were applied decreasing NADPH by sample position, the CV was reduced from 3.1 - 1.1%. This correction was therefore adopted for all ATP analyses.

It had been questioned whether the volume of the sample in the Cobas Bio cup could influence the volume of the sample delivered by the centrifugal analyses for final analysis. The effect of the sample volume in the Cobas Bio cup on ATP and CP results was therefore investigated. One sample containing ATP and CP was distributed over 24 Cobas Bio sample cups: 200  $\mu\text{l}$  in the odd and 500  $\mu\text{l}$  in even numbered cup positions. There were no significant differences in the results of ATP or CP between the two different sample volumes ( $P > 0.05$ , paired  $t$  - test). An 80 second incubation and a 300  $\mu\text{l}$  sample volume in each cup on the Cobas Bio rotor was therefore adopted.

The within assay variability was determined by repeated analysis (24 times) of the well-mixed pooled supernatant of a homogenated heart sample. The CV for ATP and CP were 1.14% and 0.48% respectively. The between batch CV for ATP [mean (sem) = 369 (7)  $\mu\text{M}$ ,  $n = 8$ ] was 5% and for CP [mean (sem) = 231 (4)  $\mu\text{M}$ ,  $n = 11$ ] was 6%.

#### 2.10.4 LACTATE

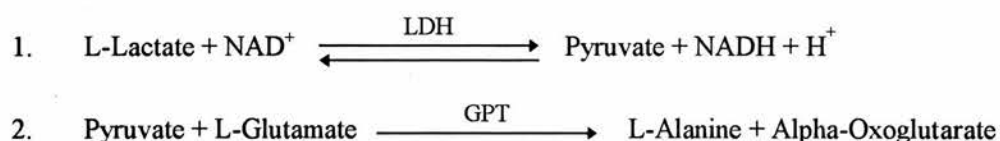
##### a). Lactate in perfusate

A fully enzymatic kit (kit No. 256773, made by Boehringer Mannheim) was used for the analysis on the Cobas Bio centrifugal analyser. The reagents in the kit were modified to use 40% less  $\text{NAD}^+$ , as the kit was designed for clinical samples that have a much





greater concentration of lactate. The principle of the coupled assay is shown by the following reactions:



The conversion of lactate to pyruvate was catalysed by lactate dehydrogenase (LDH). In the presence of excess  $\text{NAD}^+$ , NADH was produced. The NADH was measured spectrophotometrically (340 nm; 37 °C). The equilibrium of the second reaction catalysed by glutamic pyruvic transaminase (GPT), unlike that of the first, lies far to the right and this drives the first reaction to completion.

The standard: 1 mM L-lactate (in triplicate) was used. Precinorm S was used as a quality control (nominal mean concentration 880  $\mu\text{M}$ ). The within essay CV of lactate was 1.8% ( $n = 24$ ). It was determined by repeated analysis of Precinorm S within one analysis batch. The between batch CV was 5% [895 (13)  $\mu\text{M}$ ,  $n = 13$ ].

#### **b). Tissue Lactate**

The enzymatic method used for the determination of tissue lactate levels was the same as that employed for lactate concentration in the perfusate. Lactate was extracted from the freeze dried heart and liver as described in section 2.9. Samples prepared for the ATP and CP assay were used for tissue lactate concentrations.

#### **2.10.5 The Effects of Cooling Methods on Myocardial Metabolites**

An attempt was made to get an indication of ischaemic glycogen utilisation by measuring the glycogen levels in the ischaemic and non-ischaemic myocardium. To separate non-ischaemic myocardium, a dye (1% Evans blue) was perfused through the aorta.

Unfortunately it was difficult to identify the ischaemic myocardium in freeze clamped hearts (geometry is distorted). Other methods to cool the heart rapidly were therefore examined to see if these would stop any metabolic activity and yet allow the dissection of the ischaemic and non-ischaemic myocardium without altering the myocardial levels of glycogen, ATP, CP and free glucose. Four methods were compared: the effect of cooling the heart by freeze clamping, immersion into liquid nitrogen, freezing on dry ice and cooling on ice.

The results (Table 2.8) showed that immersing the heart in liquid nitrogen or placing on dry ice is adequate for the measurement of glycogen, free glucose and ATP, but not for measuring CP. Both ATP and CP levels declined over 60 - 90 minutes in hearts cooled on ice. Even glycogen levels showed a marked decline. The hearts cracked in liquid nitrogen.

**Table 2.8** Myocardial metabolites from isolated perfused hearts of the rats cooled by different methods.

| Metabolites<br>( $\mu\text{mol g}^{-1}$ dry wt) | Freeze clamped<br>(n = 7) | Liquid nitrogen<br>(n = 5) | Dry ice<br>(n = 7) | Ice<br>(n = 6) |
|---|---------------------------|----------------------------|--------------------|----------------|
| Glycogen  | 111.2 (5.5)               | 109.2 (6.4)                | 106.7 (8.2)        | 56.5 (11.3)**  |
| Free glucose                                    | 8.9 (0.4)                 | 11.6 (0.7)*                | 9.8 (0.5)          | 10.9 (1.9)     |
| ATP   | 22.2 (0.4)                | 23.8 (0.6)                 | 22.0 (0.9)         | 12.1 (2.4)**   |
| CP  | 18.6 (0.6)                | 15.2 (1.0)*                | 10.3 (1.8)**       | 2.6 (0.5)***   |

Isolated rats hearts undergoing 10 minutes equilibrium perfusion with standard perfusate and constant perfusion pressure. Hearts were then cooled by 4 different methods. The results are expressed as mean (sem) for the number of hearts shown (n). \*:  $p < 0.05$ , \*\*:  $p < 0.01$ , \*\*\*:  $p < 0.001$  vs. freeze clamped control, t - test.

The results show that freeze clamping gives the best results, especially for CP and free glucose. Freeze clamping was therefore adopted throughout all the experiments.

## 2.11 RADIOENZYMATIC ASSAY OF NORADRENALINE

NA concentration was measured by the radio-enzymatic method of Da Prada and Zürcher (1976). NA samples from coronary effluent (500  $\mu$ l) were kept on ice after collection and immediately stabilised by 1 : 1 addition of 0.6 N HClO<sub>4</sub> containing EGTA (2 g 100 ml<sup>-1</sup>). Samples were stored at - 40 °C until assayed.

NA was converted to the 3-O-methylated derivative normetanephrine by catechol-O-methyltransferase (COMT). The COMT was prepared from rat liver by Margaret Millar, a technician in our department. It can remain stable for up to 6 months when maintained at - 20 °C (Forfar, 1985). The formation of normetanephrine was carried out in the presence of a tritium labelled methyl-group donor, <sup>3</sup>H-methyl-S-adenosyl methionine (<sup>3</sup>H-SAM, Amersham International, Amersham, UK). The normetanephrine was purified by selective ion pair extraction with tetraphenylborate, separated by thin layer chromatography. It was subsequently oxidised to vanillin which was counted in a liquid scintillation counter (LKB 1217 RACKBETA, Pharmacia. Sweden).

Assay blanks were typically in the range 30 - 50 c.p.m. and were determined with an inter-assay CV of 17%. The standards (Sigma) used were 1.2 and 12 pM and the error was smaller (CV of 7%).

## 2.12 STATISTICAL ANALYSIS OF RESULTS

MINITAB statistics package (CLE.COM Ltd, Edgebaston) was used for all statistical analyses. The following methods were used:

1. For data from a normal distribution, one or two way analysis of variance was used to indicate significant differences in data from groups. Follow-up analysis was carried out using an unpaired t - test.
2. To indicate a significant difference in the incidence of VF or VT, a Chi-square test was used. However, if cells with an expected value less than five were present, a Fisher's exact test was used instead.
3. Other arrhythmia parameters such as onset and duration of VT or VF had skewed distributions and were analysed by using non-parametric Mann-Whitney test. The NA overflow results were analysed by Kruskal-Wallis test.

Results are expressed as mean with standard error of mean (sem). For all statistical analyses, the null hypothesis was rejected at the 95% level. That is,  $p < 0.05$  was considered significant.

## **CHAPTER 3**

### **The Effect of Insulin and Potassium on Anoxia Induced Noradrenaline Overflow**

### 3.1 BACKGROUND

The protective effects of myocardial glycogen and sympathetic activity on the ischaemic myocardium have been discussed in Chapter one. Glucose-insulin-potassium infusion is often used to raise myocardial glycogen. Insulin itself has been shown to play a crucial role in the regulation of sympathetic activity. A study of isolated mesenteric arteries of Sprague-Dawley rats has shown that the NA overflow induced by electrical stimulation was decreased by insulin (Shimosawa *et al.*, 1992). On the other hand, the effects of insulin deficiency on cardiac NA overflow was studied by Ganguly and colleagues (1987). They found that streptozotocin-induced diabetic rats had increased NA levels in various organs including the heart. In addition, the increase in cardiac NA could be reversed by treatment with insulin. They concluded that cardiac NA in the diabetic rat was maintained at a higher level partly due to an increased uptake of circulating NA by adrenergic nerve terminals. Furthermore, high doses of catecholamines produced myocardial cell damage (Rona *et al.*, 1959) and the pattern of injury is similar to that seen in diabetic animals (Seager *et al.*, 1984). However, the role of insulin in anoxia-evoked NA overflow remains unknown, and it was therefore investigated in the present study by using the isolated heart of the rat.

### 3.2 EXPERIMENTAL PROTOCOL

Rats were anaesthetised with pentobarbitone. Hearts were isolated and perfused at constant flow rate ( $10 \text{ ml min}^{-1}$ ) throughout the experiments. Two hearts were mounted in chambers simultaneously in each run. One of the two served as a control.

In all experiments, an initial 30 minutes normoxic perfusion with a standard perfusate (described in Chapter 2) containing 11 mM glucose and 2.5 mM  $\text{Ca}^{++}$  was performed. Perfusate contained 4 mM  $\text{K}^+$  in 'normal' potassium experiments and 3 mM  $\text{K}^+$  in 'low' potassium experiments. For the experimental hearts, 2000  $\mu\text{U l}^{-1}$  insulin was added to the perfusate before or during anoxia. The normoxic pre-perfusion was followed by 40 minutes glucose free anoxic perfusion.

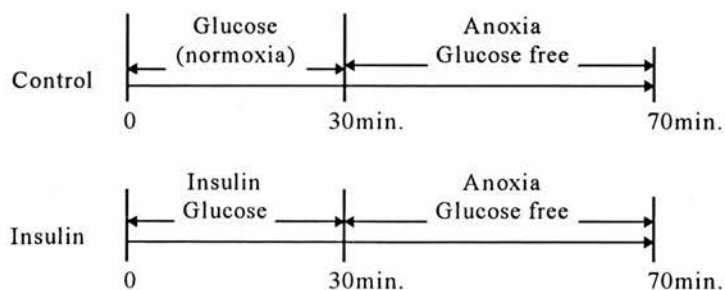
The time control of anoxia was not performed as the interests was lie in the initial myocardial glycogen levels on anoxia induced NA release. It is known that anoxia itself does not cause NA release if glucose is present in the perfusate.

Hearts were randomly assigned to each treatment. The following comparisons were made:

### 3.2.1 Normal $\text{K}^+$ (4 mM)

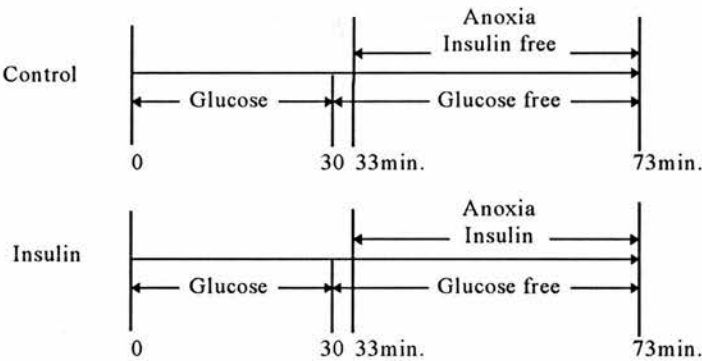
#### A. The effects of insulin on anoxic NA overflow

Insulin was used to load myocardial glycogen levels before anoxia. The experiment was divided into two groups: perfusate containing insulin 30 minutes before anoxia ( $n = 8$ ) vs. insulin free control hearts ( $n = 8$ ).



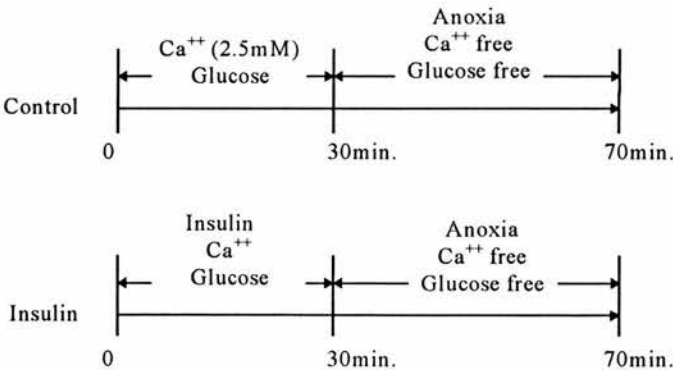
**B. The effects of insulin on NA overflow during anoxia**

The experiment was divided into two groups: anoxic perfusion in the presence of insulin (n = 10) vs. insulin free control hearts (n = 9). To exclude the effects of perfusate glucose on insulin function, the hearts underwent 3 minutes glucose free perfusion just before anoxia.



**C. The effects of insulin on anoxic Ca<sup>++</sup>-independent NA overflow**

Two groups were included in the experiment: perfusate containing insulin 30 minutes before anoxia (n = 9) vs. insulin free control hearts (n = 8). Perfusion with anoxic perfusate containing EGTA and no Ca<sup>++</sup>.

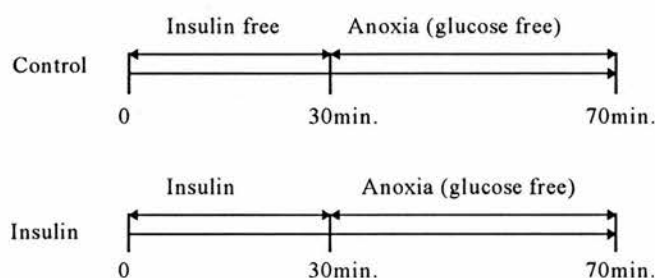




### 3.2.2 Low $K^+$ (3 mM)

#### The effects of insulin on anoxic NA overflow

Hearts were perfused with a buffer containing 3 mM  $K^+$  throughout the experiment. Insulin was added to the perfusate 30 minutes before anoxia ( $n = 8$ ) and the results were compared with insulin free control hearts ( $n = 8$ ).



The hearts were perfused until the end of 40 minutes anoxia. Lactate production, anoxic NA overflow were determined. To reduce the cost of the experiments, the initial NA levels were not measured in normal  $K^+$  A group and low  $K^+$  perfused hearts (From the results of other groups we know that the initial NA levels are less than 10 pmol dry  $g^{-1}$  in mean). The total NA overflow during anoxia (area under curve) was calculated assuming the initial NA = 0 pmol  $g^{-1}$  dry wt.

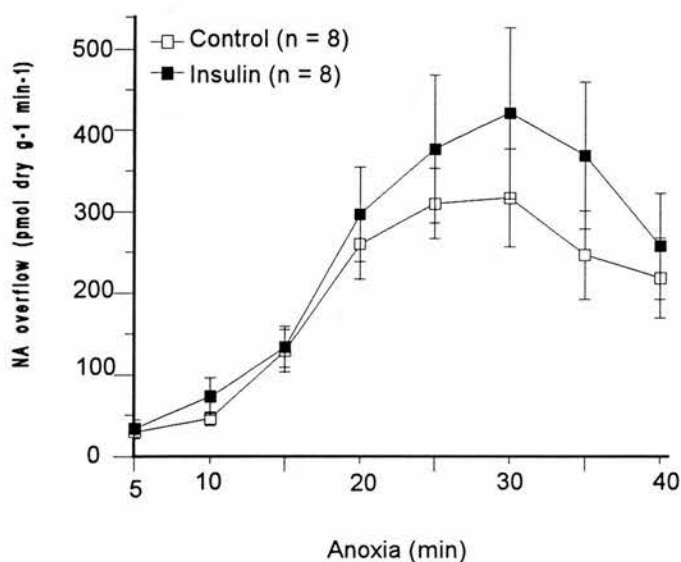
### 3.3 RESULTS

#### 3.3.1 Normal K<sup>+</sup>

##### A. The effects of insulin on anoxic NA overflow

Pre-perfusion of the heart with insulin did not significantly affect the total anoxic NA overflow ( $p > 0.05$ , Kruskal-Wallis test). The total NA overflow (areas under the curve,  $\text{pmol}^{-1} \text{g}^{-1} \text{dry wt } 40 \text{ min}^{-1}$ ) were 7243 (976) (control) and 9168 (1940) (insulin). The peak NA levels ( $\text{pmol g}^{-1} \text{dry wt min}^{-1}$ ) were 368 (54) (control) and 462 (101) (insulin) ( $p > 0.05$ , Kruskal-Wallis test). The time to the peak of NA overflow (min) was at 28 (2) (control) and 29 (2) (insulin) ( $p = 0.4$ , Fig. 3.1).

**Fig. 3.1** Noradrenaline overflow from anoxic hearts in the absence of glucose. The experimental hearts were perfused with insulin before anoxia. Each point represents the mean with vertical lines indicating s.e.m..



The total coronary lactate production during anoxia (area under the curve) was significantly higher in insulin pre-treated hearts. There were no significant differences in the peak lactate levels and the time to the peak lactate production when compared with control hearts (Table 3.1).

**Table 3.1** Coronary lactate production from anoxic hearts.

| Lactate production  | Control (n = 8) | Insulin (n = 8) |
|---|-----------------|-----------------|
| Area under curve ( $\mu\text{mol g}^{-1}$ dry wt 40 min <sup>-1</sup> ) | 294 (14)        | 401 (28)**      |
| Peak level ( $\mu\text{mol g}^{-1}$ dry wt min <sup>-1</sup> )          | 63 (3)          | 68 (5)          |
| Time of peak lactate (min)  | 1.5 (0.2)       | 1.5 (0.2)       |

The experimental hearts were perfused with insulin before anoxia. \*\*  $p < 0.01$  vs. control, analysis of variance. The results are expressed as mean (sem) for the number of hearts shown (n).

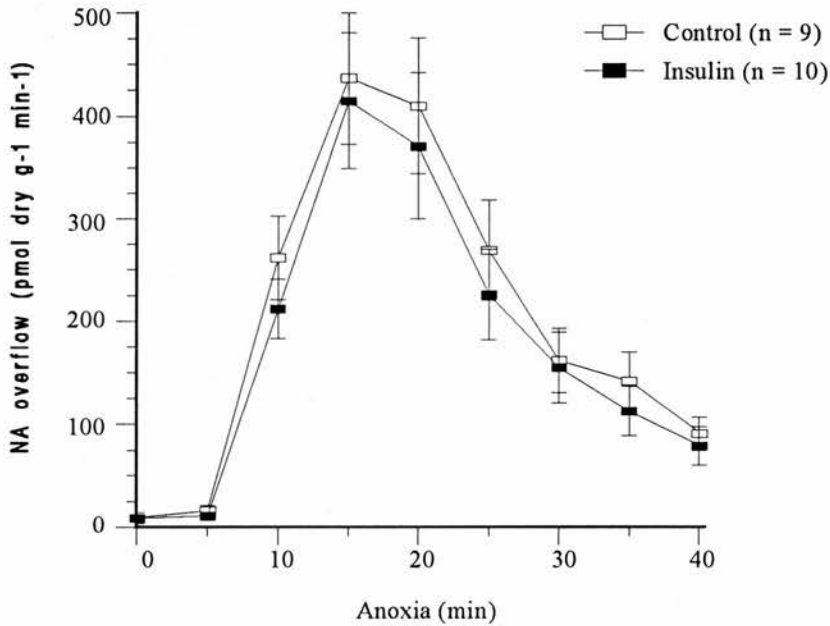
## B. The effects of insulin on NA overflow during anoxia

When insulin was only added to the perfusate during anoxia (to prevent glycogen loading) in the experimental hearts, this did not affect any of the parameters measured when compared with insulin free control hearts ( $p > 0.05$ , analysis of variance).

The NA overflow during anoxia is shown in Fig. 3.2. Total anoxic NA overflow (area under the curve,  $\text{pmol g}^{-1}$  dry wt 40 min<sup>-1</sup>) during anoxia was 7715 (1276) in the insulin treated hearts and 8716 (1377) in the control hearts. The peak NA overflow ( $\text{pmol g}^{-1}$  dry wt min<sup>-1</sup>) was 438 (74) in the insulin treated hearts and 449 (67) in the control hearts. Peak NA overflow occurred at similar times: 16 (0.7) minute in the insulin treated hearts and 17 (0.8) minute in the control hearts.

**Fig. 3.2** Noradrenaline overflow from the anoxic heart in the absence of glucose. The experimental hearts were perfused with insulin during anoxia.

Each point represents the mean with vertical lines indicating s.e.m.. There were no significant difference between insulin and control hearts ( $p > 0.05$ , analysis of variance).

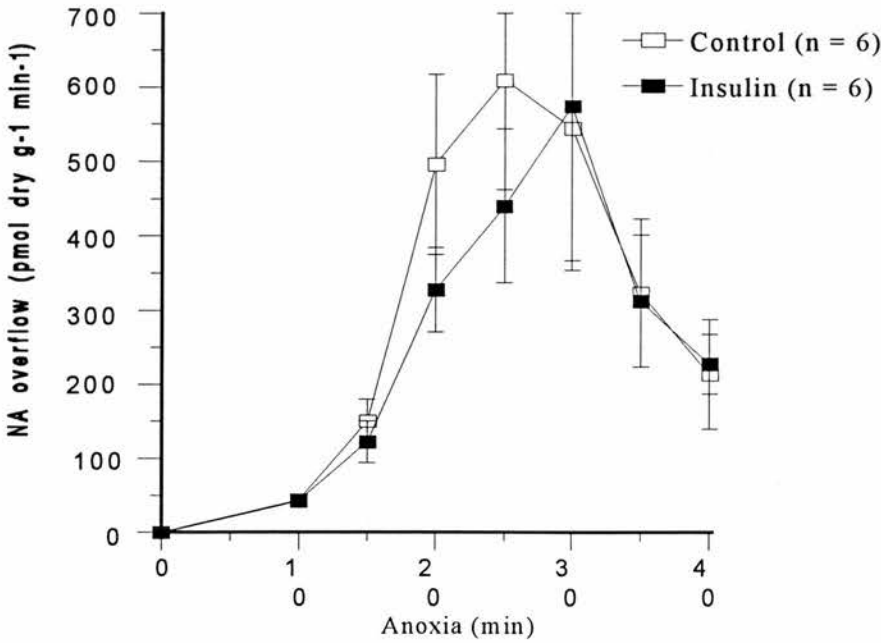


Insulin did not alter total coronary lactate production during anoxia (areas under the curve,  $\mu\text{mole g}^{-1}$  dry wt 40 min<sup>-1</sup>): 297 (30) in the insulin treated hearts and 286 (21) in the control hearts. Nor did insulin alter the time of peak lactate production: 1.4 (0.2) minute in the insulin treated hearts and 1.3 (0.2) minute in the control hearts. The peak lactate levels ( $\mu\text{mole g}^{-1}$  dry wt min<sup>-1</sup>) were similar: 58 (3) in the insulin treated hearts and 51 (1) in the control hearts.

**C. The effects of insulin on anoxic  $\text{Ca}^{++}$ -independent NA overflow.**

Perfusing the hearts with insulin for 30 minutes before anoxia did not significantly affect  $\text{Ca}^{++}$ -independent NA overflow during anoxia (Fig. 3.3). Total NA overflow (area under the curve,  $\text{pmol g}^{-1} \text{ dry wt } 40 \text{ min}^{-1}$ ) was 11040 (2805) in the control hearts and 9306 (2395) in the insulin pre-treated hearts ( $p = 0.4$  vs. control, Kruskal-Wallis test). The peak level of NA overflow ( $\text{pmol g}^{-1} \text{ dry wt min}^{-1}$ ) was 646 (141) in the control hearts and 608 (199) in the insulin pre-treated hearts ( $p = 0.5$  vs. control, Kruskal-Wallis test). The time of peak NA overflow (min) was: 23 (1) in the control hearts and 27 (2) in insulin pre-treated hearts ( $p = 0.1$ , t - test).

**Fig. 3.3** Noradrenaline (NA) overflow from anoxic hearts in the absence of  $\text{Ca}^{++}$  and glucose. The experimental hearts were perfused with insulin before anoxia. Each point represents the mean with vertical lines indicating s.e.m..



Insulin pre-treated hearts had higher total lactate production during anoxia and there was a delay in the peak of lactate production when compared with control hearts (Table 3.2).

**Table 3.2** Coronary lactate production from anoxic hearts.

| Anoxic lactate production   | Control (n = 8) | Insulin (n = 9) |
|---|-----------------|-----------------|
| Area under the curve ( $\mu\text{mol g}^{-1}$ dry wt 40 min <sup>-1</sup> ) | 214 (10)        | 358 (13)***     |
| Peak level ( $\mu\text{mol g}^{-1}$ dry wt min <sup>-1</sup> )              | 14 (0.4)        | 15 (0.8)        |
| Time of peak lactate (min)  | 6 (0.7)         | 17 (2.0)***     |

Hearts were perfused in the absence of glucose and calcium during anoxia. The experimental hearts were perfused with insulin before anoxia. The results are expressed as mean (sem) for the number of hearts shown (n). \*\*\*:  $p < 0.001$  vs. control, t - test.

#### D. The effects of $\text{Ca}^{++}$ on lactate production during anoxia

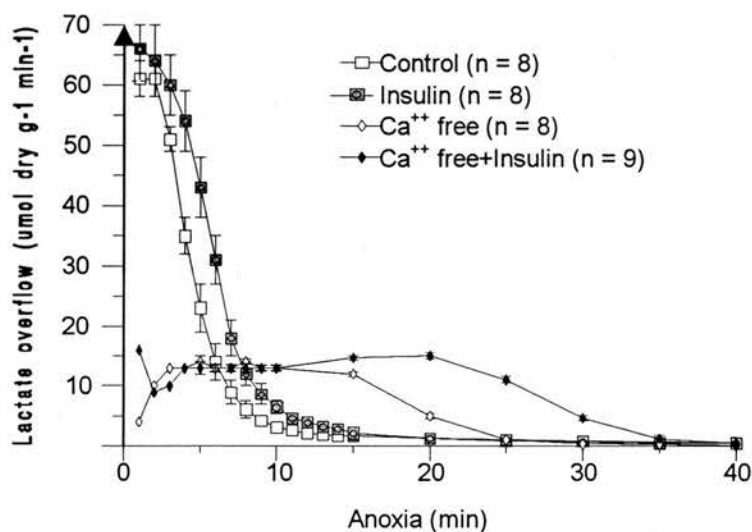
As discussed above, in the same experiments the anoxic perfusate did not contain  $\text{Ca}^{++}$ . The effect of  $\text{Ca}^{++}$  omission on lactate production during anoxia can therefore also be examined. The production of lactate during anoxia showed a different pattern between these and the earlier experiments (Fig. 3.4).

**Fig. 3.4** Lactate overflow from anoxic hearts in the absence of glucose.

In the presence of  $\text{Ca}^{++}$  with and without insulin before anoxic perfusion.

In the absence of  $\text{Ca}^{++}$  with and without insulin pre-anoxic perfusion.

Each point represents the mean with vertical lines indicating s.e.m..



The total coronary lactate production during anoxia was lower in the heart perfused without  $\text{Ca}^{++}$  than in those perfused with  $\text{Ca}^{++}$  during anoxia. There were no significant differences in the total lactate production (area under curve) in insulin pre-treated hearts both with or without  $\text{Ca}^{++}$  ( $p = 0.3$ ,  $t$ -test) (Table 3.3).

**Table 3.3** Lactate production from anoxic hearts.

| Lactate production                                     | $\text{Ca}^{++}$ 2.5 mM | $\text{Ca}^{++}$ free |
|--|-------------------------|-----------------------|
| ( $\mu\text{mol g}^{-1}$ dry wt 40 $\text{min}^{-1}$ ) | (n = 16)                | (n = 8 - 9)           |
| Control  | 294 (14)                | 214 (10)***           |
| Insulin  | 401 (28)                | 358 (13)              |

\*\*\*:  $p < 0.001$  vs.  $\text{Ca}^{++}$  perfused hearts,  $t$ -test. The hearts were the same as those in Table 3.1 and Table 3.2. The results are expressed as mean (sem) for the number of hearts shown (n).

### 3.3.2 Low K<sup>+</sup>

#### The effects of insulin on anoxic NA overflow

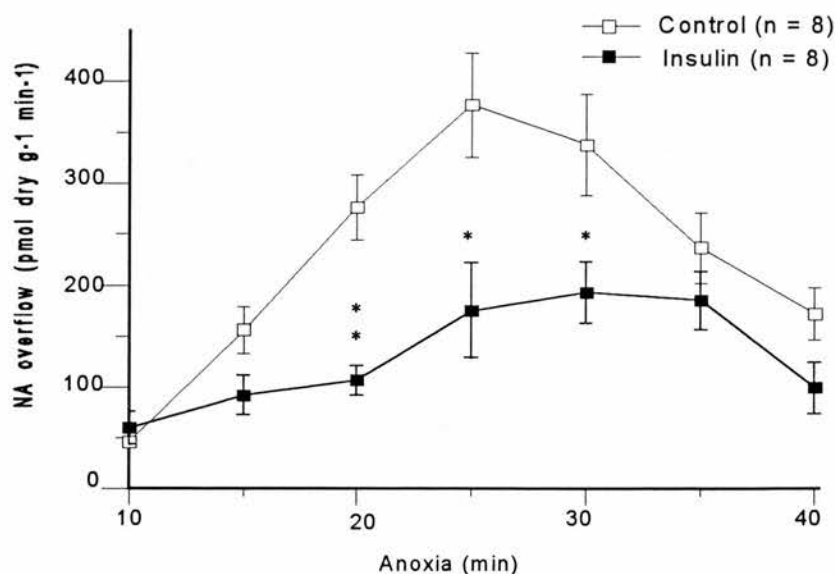
Pre-perfusion of the hearts with insulin resulted in a reduction in total NA overflow during anoxia ( $p = 0.01$ ). Insulin also delayed the time to the peak of anoxic NA overflow, and reduced the peak levels of NA overflow (Table 3.4, Fig. 3.5).

**Table 3.4** Coronary NA overflow from anoxic hearts.

| Anoxic NA overflow  | Control (n = 8) | Insulin (n = 8)         |
|---|-----------------|-------------------------|
| Area under curve ( $\text{pmol g}^{-1} \text{ dry wt } 40 \text{ min}^{-1}$ ) | 7691 (790)      | 4449 (598) <sup>+</sup> |
| Peak NA level ( $\text{pmol g}^{-1} \text{ dry wt min}^{-1}$ )                | 400 (50)        | 257 (28) <sup>+</sup>   |
| Time of peak NA (min)   | 26 (1)          | 31 (2)*                 |

Glucose free perfusion during anoxia. The perfusate contained 3 mM K<sup>+</sup> throughout. The experimental hearts were perfused with insulin before anoxia. The results are expressed as mean (sem) for the number of hearts shown (n). \*:  $p < 0.05$  vs. control, t - tests. <sup>+</sup>:  $p < 0.05$  vs. control, Kruskal-Wallis test.

**Fig. 3.5** NA overflow from anoxic hearts. The experimental hearts were perfused with insulin before anoxia. Perfusate contained 3 mM K<sup>+</sup> throughout. \*:  $p < 0.05$ ; \*\*:  $p < 0.01$  vs. control. Each point represents the mean with vertical lines indicating s.e.m..





The aim of the experiments was to investigate the influence of myocardial glycogen levels just before anoxia on anoxia-induced NA release. There was therefore no need for a time control.

Myocardial glycogen levels ( $\mu\text{mole g}^{-1}$  dry wt) were almost completely depleted at the end of 40 minutes anoxia [control: 2 (0.1), insulin 2 (0.3)]. There were no significant differences ( $p = 0.2 - 0.9$ ) between the insulin pre-treated hearts and the insulin free controls. Free glucose levels ( $\mu\text{mole g}^{-1}$  dry wt) in the myocardium were 0.3 (0.2) (control) and 0.1 (0.02) (insulin). ATP levels ( $\mu\text{mole g}^{-1}$  dry wt) were 1 (0.1) (control) and 0.1 (insulin). CP levels ( $\mu\text{mole g}^{-1}$  dry wt) were 1 (0.1) (control) and 1 (0.1) (insulin). Tissue lactate levels ( $\mu\text{mole g}^{-1}$  dry wt) were 5 (2) (control) and 5 (1) (insulin). The tissue lactate was washed out during anoxia as coronary flow was not restricted. This is in contrast to low flow or no flow ischaemia where higher tissue lactate levels are observed.

During 40 minutes glucose free anoxic perfusion, the total coronary lactate production ( $\mu\text{mole g}^{-1}$  dry wt  $40 \text{ min}^{-1}$ ) was higher in the insulin pre-treated hearts [360 (23)] than in the control hearts: [211 (12)], as expected ( $p < 0.05$ ,  $t$  - test). The peak lactate levels ( $\mu\text{mole g}^{-1}$  dry wt  $\text{min}^{-1}$ ) were 67 (4) in insulin pre-treated hearts and 54 (4) in the control hearts. The time to peak of lactate production (min) did not differ significantly between insulin pre-treated and control hearts [1.1 (0.1) vs. 1.3 (0.3)].

### 3.4 DISCUSSION

The study described has demonstrated that insulin reduces NA overflow during anoxia in low  $K^+$  (3 mM) perfused rat hearts.

It is well established that sodium pump or Na-K-ATPase plays an important role in preventing NA overflow. The inhibition of the sodium pump with ouabain can mediate the efflux of NA in the isolated perfused rat heart (Schömig *et al.*, 1988) and in chromaffin cells (Suchard *et al.*, 1982). The chromaffin cell is a conventional model for the study of sympathetic nerve terminals.

The release of NA during anoxia occurs through two mechanisms, calcium dependent and calcium independent, as discussed in the Chapter 1. The former is a depolarization related process and the latter is energy dependent. Decreased  $[K^+]$  in the perfusate can reduce calcium dependent NA overflow due to hyperpolarization of the cell membrane. The low  $[K^+]$  should not affect calcium independent NA overflow. There are  $Na^+$  pumps in the membrane of the vesicles containing NA. Insulin is known to stimulate  $Na^+$  pump. The pumps are known to play an important role in maintaining NA within the vesicles. The better function during anoxia could decrease NA overflow. This is precisely what we observed. The results suggests that the action of sodium pump activation of insulin is obvious only under low  $[K^+]$ . To confirm this finding, further study should consider  $Ca^{++}$  free perfusion during anoxia.

The information relating directly to the metabolic status of the sympathetic nerve terminals within the heart was not available. It was therefore not possible to determine whether there was a good relation between insulin, ATP production and reduced NA overflows. Nor do ATP levels indicate precisely what happens in the nerves. However

during glucose free anoxia the heart rapidly becomes quiescent so that differences in energy utilisation between neurones and myocytes, as a result of mechanical activity, are reduced. Although myocardial ATP during anoxia was not measured, the major source of ATP generated was by anaerobic glycolysis of endogenous glycogen with the production of lactate. The lactate cannot be utilised due to anoxia and can thus serve as an index of glycolytic ATP generation. The lactate overflow during anoxia could therefore be used as an indirect guide to events in the nerve terminal.

The  $\text{Na}^+\text{-Ca}^{++}$  exchanger and  $\text{Na}^+\text{-H}^+$  exchanger are not directly energy dependent. They only require a  $\text{Na}^+$  gradient across the cell membrane. However, the gradient is primarily provided by the sodium pump. The exchangers may still function for a short time after energy production is reduced or ceases. In addition, increased glycolytic ATP in insulin treated hearts could support sodium pump function for a longer time than in control hearts. Precisely as we observed, this could delay the time to peak NA levels in the insulin perfused hearts.

The sodium pump activation and stimulation of glucose transport are two independent effects of insulin. Both of these functions are energy dependent. Glycogen, which is enhanced by insulin, is the only energy source during anoxia. The increased glycolytically produced ATP in insulin treated hearts could therefore ensure that the activation of the sodium pump is observed both before and during anoxia. Clinically, glucose-insulin infusion is often used to reduce  $[\text{K}^+]_0$  and to treat patients with hyperkalaemia, while potassium-insulin infusion is used in hyperglycaemia. Nevertheless, the glycolytic ATP production did not play a decisive role in changing anoxic NA overflow in the present study.

Apart from insulin, external  $[\text{K}^+]$  could also influence glucose utilisation and lactate production according to Newman *et al.* (1991). By using an ischaemic model of the brain *in vitro*, they revealed that increasing  $[\text{K}^+]$  in the buffer resulted in increased

glucose utilisation and tissue lactate production. They concluded that a high  $[K^+]$  may exert a dual influence on tissue metabolism, not only stimulating glucose utilisation by inducing depolarisation but also by influencing the removal of metabolic products.

The protective effects of insulin in the 3 mM  $K^+$  perfused heart is significant for experimental studies. In the models of ischaemia-induced arrhythmias such as the isolated perfused rat heart, the perfusate  $[K^+]$  is often deliberately reduced to induce a higher incidence of arrhythmias.

The anoxic total lactate overflow was lower in the absence of  $Ca^{++}$  than in its presence. However when hearts had been perfused with an insulin buffer before anoxia, removal of  $Ca^{++}$  did not reduce lactate production. This suggests that the residual insulin in the hearts promotes glycolysis during anoxia. Whether  $Ca^{++}$  effects the washout of insulin is not clear.

Hof *et al.* (1988) investigated the effects of increasing  $[K^+]_0$  during neuronal activity on  $^3H$ -glycogen levels. They found that  $K^+$  stimulates the hydrolysis of  $^3H$ -glycogen in mouse cerebral cortical slices in a time- and concentration- dependent manner. Over 70% of the maximal effect is reached within 30 seconds. Significant  $^3H$ -glycogen hydrolysis occurs at 5 - 12 mM  $[K^+]_0$  during neuronal activity (field stimulations). They also showed that  $K^+$ -evoked glycogenolysis is  $Ca^{++}$ -dependent. It was concluded that  $K^+$  released in the extracellular space by active neurons may promote the mobilisation of energy substrates and therefore plays a role in the coupling between neuronal activity and energy metabolism. The present study suggests that glycogenolysis evoked by  $K^+$  in the heart during anoxia follows the same process ( $Ca^{++}$  dependent) (Fig. 3.4).

### **3.5 SUMMARY**

The results of the study suggest that insulin can prevent anoxic NA overflow in isolated rat hearts. This effect only occurred in low potassium, not in normal potassium perfused hearts. The mechanism is unknown, but the activation of  $\text{Na}^+$  pump by insulin could be the reason.

## **CHAPTER 4**

# **The Effects of Anaesthesia and its Conditions on Myocardial Glycogen and its Variability**

## 4.1 INTRODUCTION

Anaesthetised rats are often used for experiments in which the determination of myocardial glycogen content is important, but little is known about the changes in myocardial glycogen levels induced by anaesthesia. The alteration of plasma glucose and insulin by anaesthetics (details in Chapter one) may have an effect on myocardial glycogen.

This study compared the effects of three different anaesthetics on myocardial metabolism in rats. In perfusion studies, we have shown that marked variations in myocardial glycogen levels (after 10 minutes perfusion) occur in the fasted, pentobarbitone anaesthetised rat (coefficient of variation: 35%). This can be caused by the anaesthetic which can inhibit respiration and hence induce intermittent hypoxia (arterial  $pO_2$  was 39 - 68 mmHg). It is therefore important to compare the effect of extra oxygen supply during the induction of anaesthesia on myocardial glycogen levels and its variability.

It is well established that pentobarbitone itself can stimulate sympathetic drive and increase heart rate. As the anaesthesia requires an injection to administer, the related stress could also activate myocardial glycogenolysis.

Kvetnansky and colleagues (1978) reported that even gentle handling of rats causes stress to the animals. To reduce the stress caused by handling and injection of the rats, a direct comparison between pentobarbitone and gaseous anaesthesia is not possible. Therefore the use of pentobarbitone injection in halothane anaesthetised rats as a way to reduce injection stress was examined. It was hoped that the results would allow development of a model in which myocardial glycogen levels were predictable and highly reproducible.

## 4.2 EXPERIMENTAL PROTOCOL

The study consisted of four randomised groups of rats using different methods of anaesthesia. The extra oxygen supply referred to as O<sub>2</sub>/CO<sub>2</sub> is a gas mixture with 95% O<sub>2</sub> and 5% CO<sub>2</sub>.

### 4.2.1 The influence of different anaesthetics

The following four methods were used:

- 1) Sodium Pentobarbitone
- 2) Halothane

Halothane (ICI Pharmaceuticals Macclesfield, Cheshire, U.K.) was chosen for its rapid induction. It is non-flammable, it is easy to administer and the given dose is easily titrated. Anaesthesia was induced using a mixture of halothane (4 - 5%) with room air. The mixture was continuously flushed at 2.5 litres per minute into a closed transparent cage of 50 x 36 x 22 cm which contained four rats each time. The overflow gas was diverted into the laboratory ventilation system via a fume cupboard.

- 3) Ether

Ether (Merck Ltd. Glasgow, U.K.) anaesthesia was induced by placing a rat on a metal grid in a 2.7 litre glass dessicator. Gauze pads soaked in peroxide-free diethyl ether were placed on the bottom of the dessicator below the metal grid.

- 4) No anaesthesia (stunned) as a control group

The rats were stunned by a blow on the head followed by cervical dislocation within seconds. The hearts were then removed.



#### 4.2.2 Stress Reduction

The aim of the experiment was to examine the effects of handling and of injection stress during the induction of anaesthesia on myocardial glycogen levels. In order to provide identical experimental conditions, a gas mixture of  $O_2/CO_2$  was used during anaesthesia in all rats.

1). Halothane with  $O_2/CO_2$  followed by pentobarbitone with  $O_2/CO_2$

In order to examine whether intraperitoneal injection (ip.) itself could affect the results, anaesthesia was induced by halothane followed by the administration of pentobarbitone. Rats inhaled halothane for a very short duration (3 - 4 min). As soon as the rat lost its righting reflex, pentobarbitone at  $60 \text{ mg kg}^{-1}$  ip. was administered. To prevent transient hypoxia, the rat was then placed in a cage, which was continuously flushed with a mixture of  $O_2/CO_2$ .

2). Pentobarbitone with  $O_2/CO_2$  as a control group

Some rats receiving pentobarbitone ip. were also placed in an  $O_2/CO_2$  atmosphere to provide identical experimental conditions with the halothane + pentobarbitone treated rats.

#### 4.2.3 The interaction between anaesthesia and fasting on myocardial glycogen levels.

Rats were anaesthetised with either pentobarbitone with  $O_2/CO_2$  or halothane with  $O_2/CO_2$ . In the experimental group, rats were starved for 24 or 48 hours before anaesthesia.

#### 4.2.4 Extra oxygen supply

In order to prevent hypoxia due to the depression of the central respiratory drive by anaesthetics, rats were placed in a transparent plastic covered cage, which was continuously flushed with O<sub>2</sub>/CO<sub>2</sub> at 2.5 litres per minute during the induction of anaesthesia with pentobarbitone or halothane. Pentobarbitone anaesthetised animals may be dependent on peripheral hypoxic respiratory drive which can be further depressed by oxygen. However, the 5% CO<sub>2</sub> in the oxygen mixture can limit this effect.

After the animal was anaesthetised, the abdomen was opened rapidly by making a transverse incision. An abdominal aortic blood sample of 2 ml was taken. The heart and then the liver were removed and freeze clamped for later analysis. The isolated heart was perfused (in a pressure-controlled Langendorff system) for 10 minutes with perfusate containing 5.5 mM glucose before being freeze clamped. Myocardial free glucose, lactate, ATP and CP levels were measured to determine the possible cause of the differences in myocardial glycogen levels.

All experimental studies were conducted between 9 to 11 a.m. to minimise any confounding effects that diurnal variations may have on tissue glycogen concentrations (Conlee *et al.*, 1976; Bockman *et al.*, 1971; Segel *et al.*, 1975).

### 4.3 RESULTS

#### 4.3.1 The effects of anaesthetics on myocardial glycogen

The time taken to achieve full anaesthesia varied ( $p < 0.01$ ), virtually instantaneously in stunned rats, 10 (1) minutes in ether, 17 (1) minutes in halothane and 28 (6) minutes in pentobarbitone anaesthetised rats.

The lower plasma pH,  $pO_2$  levels, higher  $pCO_2$  values (Table 4.1) and the longer time to induce anaesthesia were noted in pentobarbitone and halothane treated rats when compared with those treated with ether. However there were no correlations between the plasma  $pCO_2$  and  $[H^+]$  in either pentobarbitone ( $r = -0.16$ ,  $p > 0.1$ ) or halothane ( $r = 0.5$ ,  $p > 0.1$ ) treated rats. In ether treated rats, there was a correlation between the plasma  $pCO_2$  and  $[H^+]$  ( $r = 0.99$ ,  $p < 0.01$ ). Blood gas could not be determined in the rats that were stunned.

**Table 4.1** The abdominal aortic blood gas results of the rats after anaesthesia.

| Blood Gas      | Pentobarbitone (n = 8) | Halothane (n = 8) | Ether (n = 7) |
|----------------|------------------------|-------------------|---------------|
| pH             | 7.32 (0.01)            | 7.20 (0.01)***    | 7.44 (0.03)** |
| $pO_2$ (mmHg)  | 69 (8)                 | 83 (9)            | 274 (64)**    |
| $pCO_2$ (mmHg) | 51 (3)                 | 65 (5)*           | 42 (3)*       |

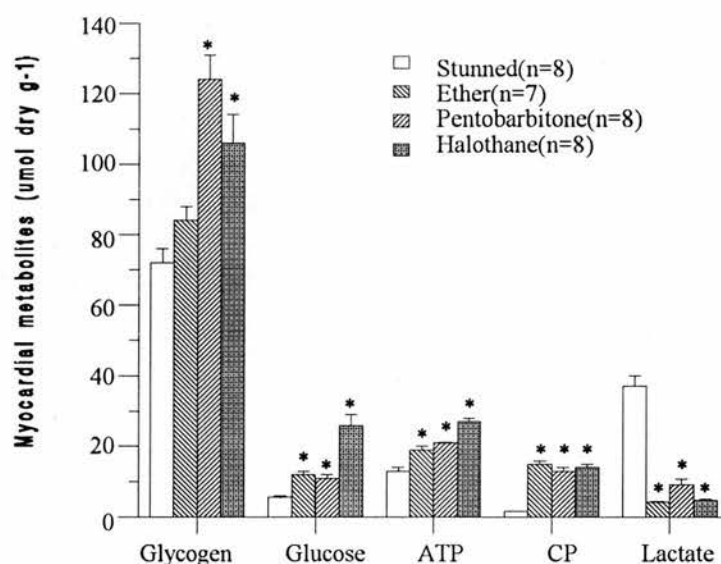
Hearts were the same as Fig 4.1. The results are expressed as mean (sem) for the number of rats shown (n). \*:  $p < 0.05$ ; \*\*:  $p < 0.01$  \*\*\*:  $p < 0.001$  vs. Pentobarbitone treated rats (Mann-Whitney test).

Multiple comparison of results showed that myocardial glycogen levels were higher ( $p < 0.05$ ) in pentobarbitone [mean difference (sem):  $52 (6) \mu\text{mol g}^{-1}$  dry wt, 95% confidence interval:  $39 - 65 \mu\text{mol g}^{-1}$  dry wt) and halothane [mean difference (sem):

29 (6)  $\mu\text{mol g}^{-1}$  dry wt, 95% confidence interval: 16 - 42  $\mu\text{mol g}^{-1}$  dry wt] anaesthetised rats than in stunned controls. Pentobarbitone treated rats also had higher ( $p < 0.05$ ) myocardial glycogen content than ether [mean difference (sem): 40 (7)  $\mu\text{mol g}^{-1}$  dry wt, 95% confidence interval: 26 - 53  $\mu\text{mol g}^{-1}$  dry wt) or halothane [mean difference (sem): 23 (6)  $\mu\text{mol g}^{-1}$  dry wt, 95% confidence interval: 10 - 36  $\mu\text{mol g}^{-1}$  dry wt] treated rats (Fig 4.1).

**Fig. 4.1** Myocardial glycogen and metabolites *in situ* in the rats.

The experimental rats were anaesthetised with either pentobarbitone, halothane or ether. Each column represents the mean with vertical lines indicating s.e.m.. \*:  $p < 0.05$  vs. stunned control (ANOVA followed by t - tests).

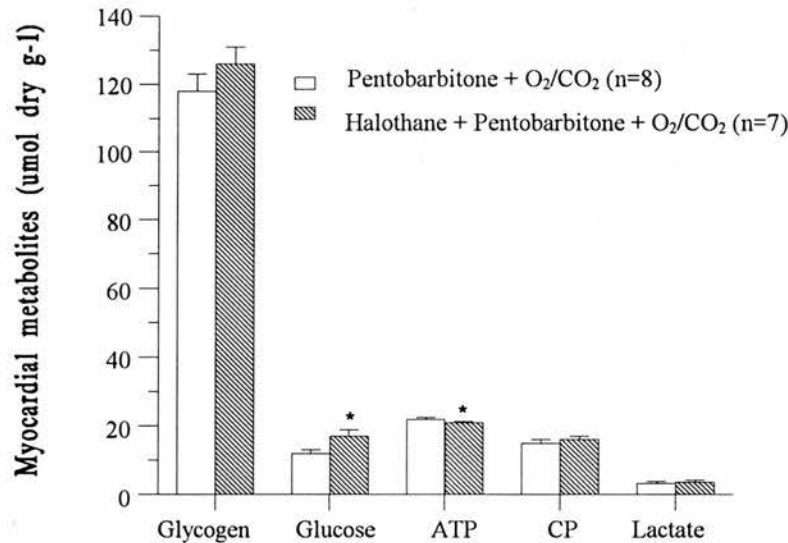


There was an inverse correlation between myocardial glycogen and free glucose levels in pentobarbitone treated rats ( $r = -0.848$ ,  $p < 0.01$ ). Rats anaesthetised with ether showed a correlation between myocardial glycogen levels and the time taken to induce anaesthesia:  $r = 0.96$ ,  $p < 0.02$ .

4.3.2 The effects of stress reduction on myocardial glycogen

To reduce handling and injection stress, the rats were anaesthetised with halothane followed by pentobarbitone or with pentobarbitone only as control. All rats were ventilated with O<sub>2</sub>/CO<sub>2</sub> during anaesthesia. There was no significant difference in myocardial glycogen levels ( $p = 0.28$ ) between the experimental and control hearts (Fig. 4.2). The times of induction anaesthesia (min) are not significantly different between halothane followed by pentobarbitone treated rats [19 (3) min] and pentobarbitone treated control rats [21 (2) min].

**Fig. 4.2** Myocardial glycogen and metabolites *in situ* in the rats. During anaesthesia, O<sub>2</sub>/CO<sub>2</sub> was supplied. The experimental rats were anaesthetised with halothane followed by pentobarbitone. Each column represents the mean with vertical lines indicating s.e.m.. \*:  $p < 0.05$  vs. pentobarbitone, t - test.



Pentobarbitone + O<sub>2</sub>/CO<sub>2</sub> treated rats had lower myocardial free glucose ( $p = 0.015$ ), slightly but significantly higher ATP ( $p = 0.022$ ), and lower plasma glucose levels ( $p = 0.006$ ).

There was a positive correlation between free glucose in the myocardium and glucose in the plasma ( $r = 0.85$ ,  $p < 0.02$ ) in halothane followed by pentobarbitone + O<sub>2</sub>/CO<sub>2</sub> treated rats. The correlation was not significant in pentobarbitone + O<sub>2</sub>/CO<sub>2</sub> treated rats ( $r = 0.63$ ,  $p < 0.1$ ).

**Table 4.2** Blood gas and plasma glucose levels in anaesthetised rats.

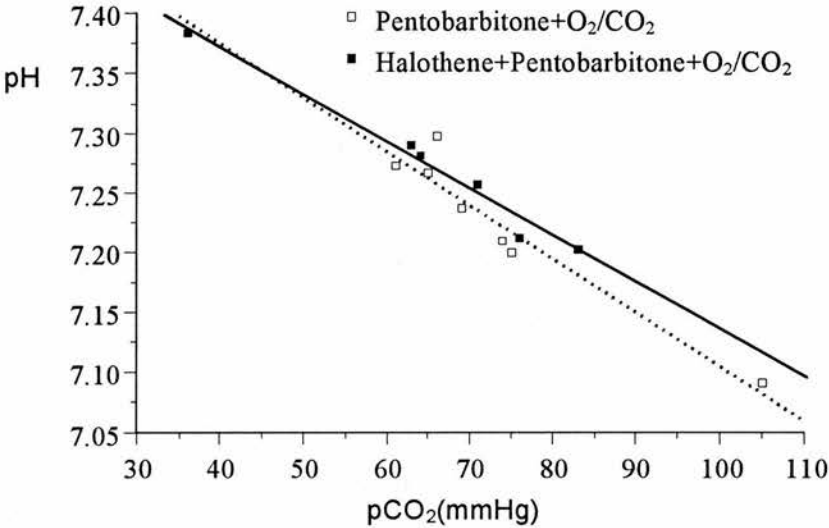
| Blood gas & plasma glucose | Pentobarbitone                            | Halothane + Pentobarbitone                |
|----------------------------|---|---|
|                            | + O <sub>2</sub> /CO <sub>2</sub> (n = 8) | + O <sub>2</sub> /CO <sub>2</sub> (n = 7) |
| pH                         | 7.23 (0.02)                               | 7.27 (0.02)                               |
| pO <sub>2</sub> (mmHg)     | 260 (45)                                  | 410 (55)                                  |
| pCO <sub>2</sub> (mmHg)    | 74 (5)                                    | 66 (6)                                    |
| Plasma glucose (mM)        | 10.9 (0.2)                                | 12.4 (0.4)**                              |

Rats are the same as those in Fig. 4.2. The arterial blood sample from abdominal aorta was taken after the rats were fully anaesthetised. Blood gas results were not influenced by the two anaesthesia methods used (pH  $p = 0.2$ , pO<sub>2</sub>  $p = 0.06$ , pCO<sub>2</sub>  $p = 0.4$ , Mann-Whitney test). The results are expressed as mean (sem) for the number of rats shown (n). \*\*:  $p < 0.01$  vs. pentobarbitone control.

There was a significant correlation between arterial pH and pCO<sub>2</sub> in both experimental and control rats: pentobarbitone + O<sub>2</sub>/CO<sub>2</sub>:  $r = -0.956$   $p < 0.01$ . Halothane + pentobarbitone + O<sub>2</sub>/CO<sub>2</sub>:  $r = -0.987$   $p < 0.01$ . (Fig. 4.3).

**Fig.4.3** The pH and pCO<sub>2</sub> of abdominal aortic blood of rats.

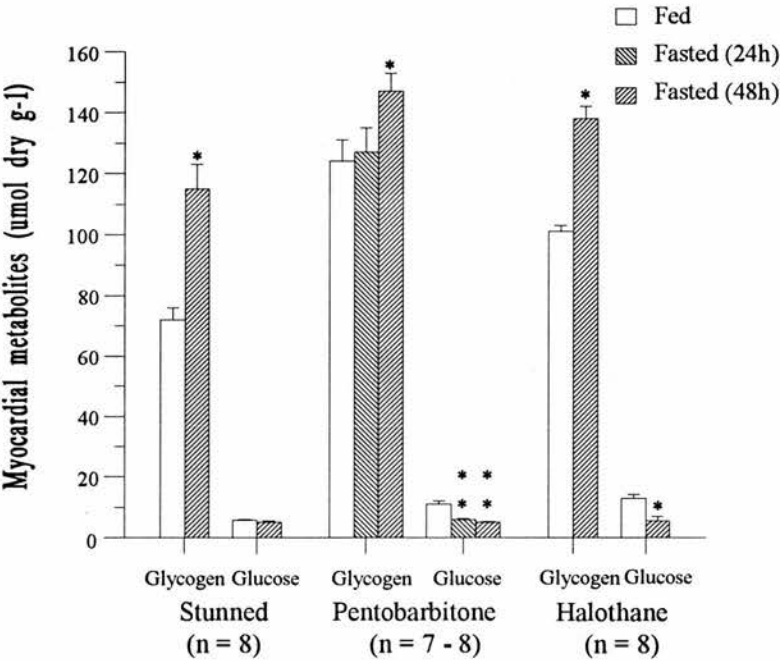
The experimental rats were anaesthetised with halothane followed by pentobarbitone. The results are mean values. The correlation between blood pH and pCO<sub>2</sub> were  $r = - 0.956$  ( $p < 0.01$ ) in pentobarbitone control, and  $r = - 0.987$  ( $p < 0.01$ ) in experimental rats. Extra O<sub>2</sub> (O<sub>2</sub>/CO<sub>2</sub>) was supplied during anaesthesia.



#### 4.3.3 The interaction between anaesthesia and fasting on myocardial glycogen levels

The effects of fasting on myocardial glycogen and metabolites were examined in rats anaesthetised by pentobarbitone or by halothane. *In situ*, myocardial glycogen levels were significantly higher ( $p < 0.05$ ) after 48 hours fasting. Myocardium free glucose was lower in (24 or 48 hours) fasted rats and also in stunned fed rats (Fig. 4.4). Myocardial ATP, CP and lactate levels were not significantly different in myocardium from fasted and fed rats.

**Fig. 4.4** Myocardial glycogen and free glucose levels of fasted rat hearts  
Rats were anaesthetised with pentobarbitone or halothane and stunned rats served as controls. Each column represents the mean with vertical lines indicating s.e.m.. \*:  $p < 0.05$ ; \*\*:  $p < 0.01$  vs. fed control, t-tests. (Hearts from pentobarbitone anaesthetised and stunned fed rats are identical as in Fig. 4.1)

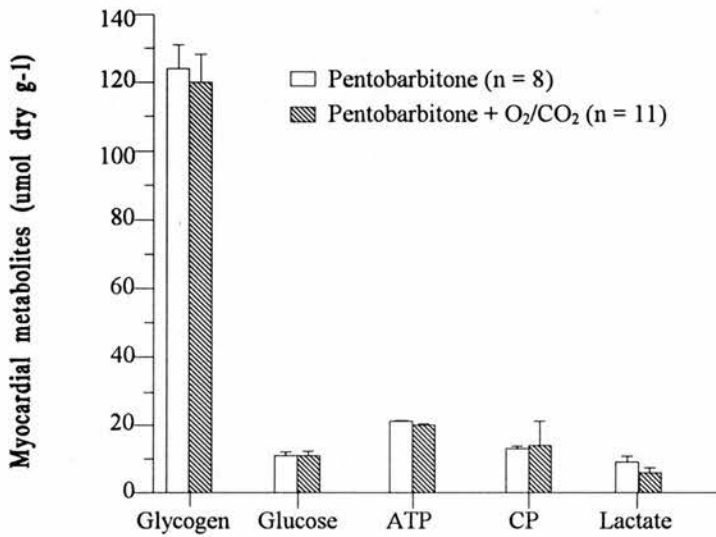


**4.3.4 The effects of +O<sub>2</sub> during anaesthesia on myocardial glycogen**

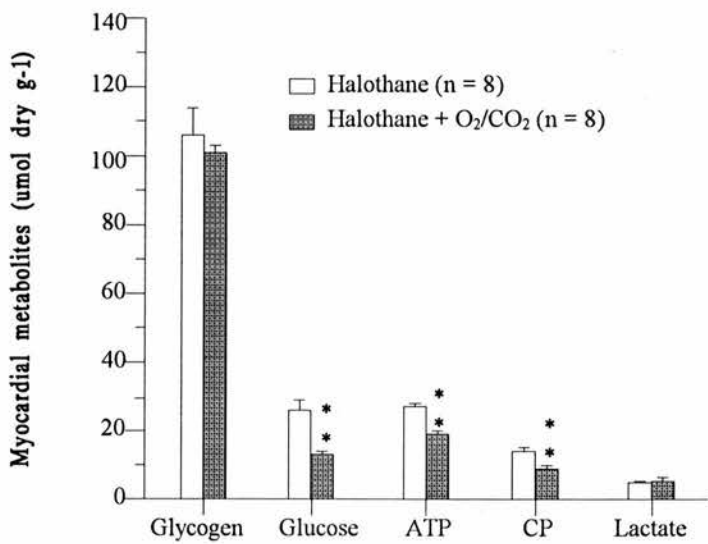
The results of myocardial glycogen levels in O<sub>2</sub>/CO<sub>2</sub> supplied rats during anaesthesia showed that in pentobarbitone anaesthetised rats, the hypoxic effects were only noticeable in 48 hours fasted rats (myocardial glycogen levels  $p = 0.026$ , Fig. 4.8 and 4.4.), not in the fed rats (Fig. 4.5 and 4.6). In halothane +O<sub>2</sub>/CO<sub>2</sub> group, the time to induce anaesthesia was shorter and there were significant reductions in myocardial free glucose, ATP and CP levels (Fig. 4.6).



**Fig. 4.5** Myocardial glycogen and metabolites in the hearts *in situ*. Rats were anaesthetised with or without supplying oxygen during anaesthesia. Each column represents the mean with vertical lines indicating s.e.m..



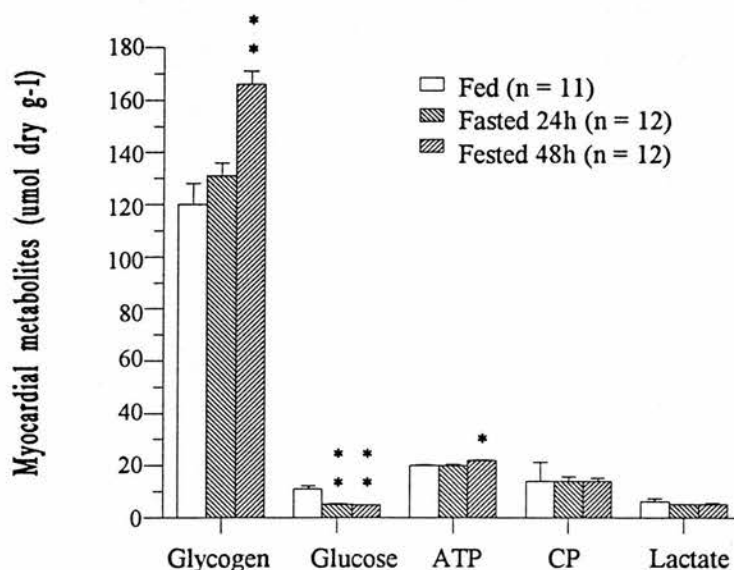
**Fig. 4.6** Myocardial glycogen and metabolites in the hearts *in situ*. Rats were anaesthetised with or without supplying oxygen during anaesthesia. Each column represents the mean with vertical lines indicating s.e.m.. \*\*:  $p < 0.01$  vs. control. t - test.



**Fig 4.7** Myocardial levels of glycogen and metabolites *in situ*.

Rats were anaesthetised with pentobarbitone. The rats of experimental hearts were fasted for 24 or 48 hours. Each column represents the mean with vertical lines indicating s.e.m..

\*:  $p < 0.05$ ; \*\*:  $p < 0.01$  vs fed control, t - test. Extra  $O_2$  was supplied during anaesthesia.

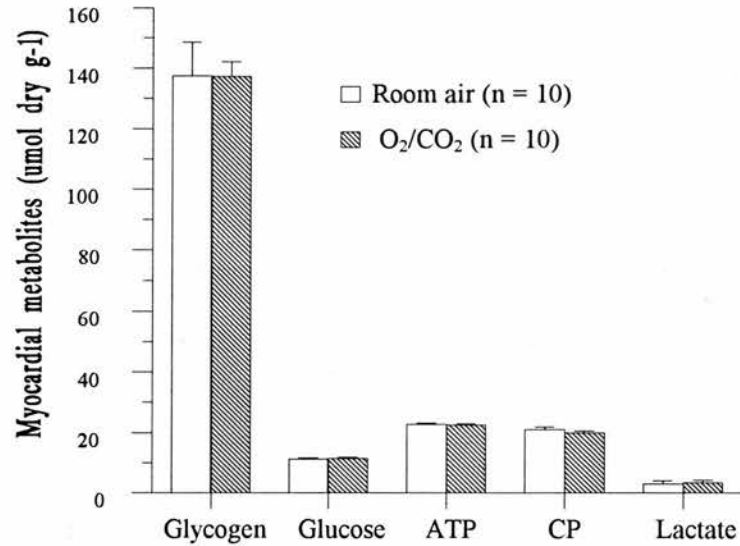


Pentobarbitone anaesthetised rats which received  $O_2/CO_2$  during anaesthesia demonstrated a higher plasma  $pO_2$  [fed: 375 (15) mmHg n = 11; 24h fasted: 276 (44) mmHg, n = 9; 48h fasted: 249 (40) mmHg n = 12.  $p < 0.01$ , t - test vs. room air control  $pO_2$ : 68 (6) mmHg, n = 20], yet they had lower plasma pH levels : 7.19 (0.02), n = 32 vs. room air control pH: 7.32 (0.01), n = 20 ( $p < 0.01$  t - test). The plasma  $pCO_2$  correlated with  $[H^+]$  ( $r = 0.83$ ,  $p < 0.01$ ) or pH ( $r = -0.82$ ,  $p < 0.01$ ).

In the room air ventilated rats, the blood gas results were not significantly different between fed and fasted animals and these averaged pH = 7.32 (0.01),  $pO_2$  = 68 (6) mmHg,  $pCO_2$  = 50 (2) mmHg; n = 20 ( $p > 0.05$ , t - test). There was no significant correlation between  $pCO_2$  and pH ( $r = 0.06$ ,  $p > 0.1$ ) or  $[H^+]$  ( $r = -0.082$ ,  $p > 0.1$ ).

The rats anaesthetised in an atmosphere of high oxygen or room air did not show a significant difference in mean glycogen levels *in vitro*. However, there was a decrease in the variability of glycogen levels in the perfused hearts from rats that had been fasted for 48 hours and given O<sub>2</sub>/CO<sub>2</sub> during anaesthesia. The s.e.mean was decreased by 55% (5 vs. 11) within the group (Fig. 4.8). Plasma lactate levels of the rats were significantly lower [933 (91)  $\mu$ M vs. 1387 (160)  $\mu$ M,  $p < 0.05$ ].

**Fig. 4.8** Myocardial glycogen and metabolites and their variability *in vitro* in relation to the prevailing atmosphere during anaesthesia. All rats were fasted for 48 hours, and anaesthetised with pentobarbitone. The experimental rats were supplied with O<sub>2</sub>/CO<sub>2</sub> during anaesthesia. Each column represents the mean with vertical lines indicating s.e.m..



Liver glycogen changes after fasting have been very well documented and were measured here as a positive control. The liver glycogen levels were depleted by 98% after one day fasting, and by 99% after two days fasting (Table 4.3).

**Table 4.3** Liver metabolites in pentobarbitone (+O<sub>2</sub>/CO<sub>2</sub>) anaesthetised rats.

| Liver metabolites (μmol g <sup>-1</sup> dry wt) | Fed (n = 6) | 24 hours (n = 7) | 48 hours (n = 7) |
|---|-------------|------------------|------------------|
| Glycogen  | 1108 (30)   | 26 (10)**        | 11 (3)**         |
| Glucose   | 30 (2)      | 16 (1)**         | 13 (1)**         |
| ATP   | 9 (0.4)     | 9 (1)            | 10 (0.3)         |
| CP  | 0.6 (0.1)   | 0.8 (0.1)        | 0.5 (0.1)*       |
| Lactate   | 7 (1)       | 3 (0.4)**        | 6 (1)            |

Rats are the same as those in Fig. 4.7. The results are expressed as mean (sem) for the number of rats shown (n). \*: p < 0.05; \*\*: p < 0.01 t - test vs. fed control.

#### 4.4 DISCUSSION

The results have shown the existence of an effect of anaesthesia on myocardial glycogen levels. However, an important limitation to this type of study is lack of suitable control. The study initially used the stunning method to obtain isolated hearts as controls. The results clearly documented that this approach may not result in an appropriate control because of the very high tissue lactate levels, lower myocardial glycogen, ATP and CP levels. Therefore, for the final conclusion, the results in stunned rats were ignored and the effect of the other types of anaesthesia were compared.

The myocardial glycogen content was higher in the pentobarbitone and halothane treated than ether treated rats. Ether is known to stimulate endogenous release of catecholamines. Hyperglycaemia can arise secondary to increased catecholamine release (details in Chapter 1). This causes stimulated myocardial glycolysis *in situ*. Merin (1970, 1970a) suggested that halothane interferes with myocardial uptake of glucose. The high myocardial glycogen content in the halothane anaesthetised rats could reflect its ganglion block reducing catecholamine release.

Even minor stress was found to cause marked changes in levels of catecholamines in rat plasma. However, we could not measure circulating catecholamine levels. Kvetnansky *et al.* (1978) has reported that simply gentle handling of rats for half a minute increased adrenaline levels of plasma about seven fold, and NA levels almost two fold above levels found in undisturbed rats. Nevertheless the precise explanation remains unknown and speculative. On the other hand, all three anaesthetics used can induce hyperglycaemia (details in Chapter 1). Animals in both pentobarbitone and halothane groups took longer to become anaesthetised and consequently this may have provided more time for glycogen synthesis.

Inhalation of halothane for a short duration, preceding the pentobarbitone treatment to reduce injection stress (Arola *et al.*, 1981; Furner *et al.*, 1972) did not show a significant effect on myocardial glycogen levels. The assumption is that rats are fully unaware of being exposed to halothane, which is rather simplistic. The higher plasma glucose levels in rats anaesthetised by halothane followed by pentobarbitone (Table 4.2) may reflect a change in plasma insulin concentrations (see Chapter 1). Whether this putative insulin mediated increase in myocardial glucose uptake is also responsible for the higher ATP levels remains to be seen.

Not surprisingly, myocardial glycogen content was increased after rats had been starved for 48 hours, and this is in agreement with others (Freminet, *et al.*, 1984; Gannon and Nuttall, 1984). The mechanism has been shown mainly to be due to increasing the level and usage of fatty acids (Opie, 1968 and 1969; Scheuer, 1967). Glycogen is then formed and conserved in the myocardium, and it cannot act as a source of circulating glucose because of the irreversible reaction catalysed by hexokinase. The higher myocardial free glucose levels in the fed rats may reflect a higher plasma glucose levels. It may also suggest that the effects of the anaesthetics on carbohydrate metabolism in the heart varies with dietary state. The extracellular free glucose is significantly different between fed and fasted rats hearts *in situ*. To make sure that this did not affect myocardial glycogen levels, the results of all glycogen concentrations shown in this thesis represent true glycogen and exclude free glucose (details see Chapter 2).

The delivery of oxygen to the heart muscle depends upon the oxygen content of the coronary arterial blood and the amount of the blood delivered to the muscle. It is widely accepted that ether stimulates respiration. Halothane and pentobarbitone can produce respiratory depression which decreases oxygen concentration in arterial blood. Anaesthetics can also decrease myocardial oxygen supply due to reduced coronary perfusion.

Fasted rats could be more sensitive to anaesthetics. The rats were therefore more likely to become hypoxic, with an increased sympathetic drive and enhanced glycolysis. These cause decreased myocardial glycogen in some rats and result in an increased variability of the glycogen levels within the group. The hypoxia during anaesthesia is very easy to correct by giving extra oxygen although oxygen may further depress respiration by eliminating hypoxic drive, particularly in pentobarbitone anaesthesia. In our data, after hypoxia has been controlled, glycogen content in the myocardium was improved in fasted rats *in situ*. The effect of hypoxia during anaesthesia could be observed even after 10 minutes of perfusion: the standard deviation of glycogen within the group was decreased by 55%, although there was no significant difference in the glycogen content of hearts from room air and oxygen supplemented rats.

However, the anaesthetic itself could also be responsible for glycogen's variability because of its hyperglycaemic effects. Furner and colleagues (1972) have shown that when pentobarbitone dose is raised from 25 to 50 mg kg<sup>-1</sup> body weight, the standard errors of plasma glucose increase 3 - 7 fold as a result of significantly increased plasma glucose levels.

The lower myocardial free glucose, ATP and CP levels in halothane +O<sub>2</sub>/CO<sub>2</sub> rats may be due to the shorter time to induce anaesthesia rather than increased glycolysis as tissue lactate did not increase. However, it is also possible that the adequate provision of oxygen might have allowed glucose derived from glycogen to be metabolised oxidatively. The present study has not been able to measure the rate of glycogen glycolysis during anaesthesia.

Rats supplied with extra-oxygen exhibited higher arterial PO<sub>2</sub> and PCO<sub>2</sub> than rats exposed to room air. This may have caused increased free radicals in the tissue. However there is no evidence that these compounds activate glycolysis.

It was noted that the plasma pH in O<sub>2</sub>/CO<sub>2</sub> supplemented anaesthetised rats were lower and were correlated negatively to pCO<sub>2</sub>. It was also noted that even without O<sub>2</sub>/CO<sub>2</sub> supplementation, the arterial blood pH and pO<sub>2</sub> were lower. Also pCO<sub>2</sub> was higher in pentobarbitone or halothane treated rats when compared with the ether treated. These results indicated effects of anaesthetics on respiration. In O<sub>2</sub>/CO<sub>2</sub> supplemented rats, the respiratory acidosis were therefore mainly due to the anaesthetics *per se*, the 5% CO<sub>2</sub> in the gas, and the oxygen gas which depressed respiration severely by removing hypoxic drive.

These differences in arterial blood gases and acid-base status did not appear to have any effect on the myocardial glycogen levels, as the glycogen contents found in the added oxygen and room air groups were similar ( $p = 0.468$ ). Opie (1965) reported that, in the isolated rat heart, a low pH (pH = 7.1) did not affect the ATP, ADP and AMP levels, and it also did not increase glycolysis or glucose uptake. On the other hand, alkalosis increased glycolysis in the perfused rat heart (Opie, 1965), presumably by phosphofructokinase (PFK) activation, since optimal PFK activity occurs at pH = 8 (Scheuer and Berry, 1967).

It is well established that 24 hour fasting almost completely depletes liver glycogen stores. The results confirmed this. This may also suggest that if the effects of anaesthetics on carbohydrate metabolism in the heart varies with dietary state, then this may be linked to the alteration of plasma glucose levels through the change of liver metabolism.

As discussed in chapter 1, there is a interaction between glycogen and noradrenaline, Therefore, anaesthetics used for studies of cardiovascular sympathetic activity, such as urethane or chloralose should therefore also be examined or re-justified, as they could affect cardiac glycogen levels which may lead to different outcomes.



## **4.5 SUMMARY**

The results of the study have shown that myocardial glycogen content in anaesthetised rats can be affected by the anaesthetic used.

Sodium pentobarbitone or halothane anaesthetised rats had significantly higher myocardial glycogen content than that of rats anaesthetised by diethyl ether. Respiratory acidosis during anaesthesia did not appear to have any effect on myocardial glycogen concentrations.

Pentobarbitone is suitable for studying myocardial glycogen metabolism in fasted rats, provided intermittent hypoxia during anaesthesia is prevented.

These findings suggest that it is necessary to take the effects of different anaesthetics into account when measuring myocardial glycogen levels, or studying glycogen related problems after using general anaesthesia. The data also suggest that extra oxygen supply during anaesthesia may be used in studies of myocardial glycogen in fasted rats in order to minimise animal variability and this may help to reduce group size.

The control groups for comparing different anaesthetics are still not ideal. The use of a non-invasive model (e.g. NMR) may help to resolve this.

## **CHAPTER 5**

# **The Effects of Fasting and Glycogen on Ischaemia-Induced Ventricular Fibrillation**

## 5.1 INTRODUCTION

It is well established that fasting can increase the glycogen concentration in the hearts of rats. Schneider and Taegtmeyer (1991) reported that fasting delays myocardial cell damage after 15 minutes of total ischaemia in the isolated working rat heart. They concluded that this was due to increased myocardial glycogen content after fasting. Whether fasting can have a similarly beneficial effect on ischaemia-induced arrhythmias *in vitro* is not yet known.

The aim of this investigation was to test the hypothesis that fasting could reduce ischaemia-induced VF in the isolated perfused rat heart due to the production of ATP from myocardial glycogen stores. The metabolic changes at the onset of ischaemia were also examined to gain a better perception of glucose metabolism in the ischaemic heart.

## 5.2 EXPERIMENTAL PROTOCOL

All rats were anaesthetised with pentobarbitone. Hearts were isolated and perfused with a pressure controlled (60 cm H<sub>2</sub>O) perfusion setting. Glucose was the basal substrate in the perfusion medium. Regional ischaemia (maximum 20 min) was induced by LAD ligation. At the end of experiments, hearts were freeze clamped for the measurement of myocardial glycogen, ATP, CP and lactate.

The following experiments were performed:

### 5.2.1 Factors affecting myocardial glycogen and metabolites

The control hearts of fed rats were freeze clamped after equilibrium perfusion. Other hearts were freeze clamped after 20 minutes perfusion. The effects of insulin, glucose and perfusion period on myocardial glycogen and metabolites levels in hearts from fed rats were examined as follows:

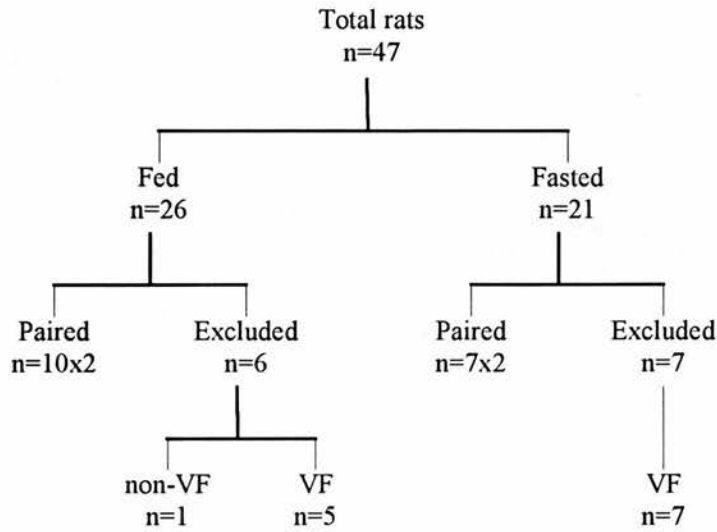
1. Control: perfusate containing 5.5 mM glucose.
2. Prolonged perfusion: perfusate containing 5.5 mM glucose.
3. Hyperglycaemia: perfusate containing 15 mM glucose.
4. Hyperinsulinaemia: perfusate containing 5.5 mM glucose and 2000  $\mu\text{U ml}^{-1}$  insulin.
5. Hyperglycaemia and hyperinsulinaemia: Perfusate containing 15 mM glucose and 2000  $\mu\text{U ml}^{-1}$  insulin.

The effects of equilibrium perfusion on fasted (48 hours) rat hearts were also checked. The hearts of both fasted ( $n = 8$ ) and fed rats ( $n = 7$  see above) were perfused with 5.5 mM glucose perfusate. The hearts were freeze clamped after equilibrium perfusion for the measurement of myocardial metabolites.

### 5.2.2 Myocardial glycogen and metabolites at the onset of VF

The total number of rats studied was 47. Of these 26 were fed *ad libitum* and 21 were fasted for 48 hours. The comparisons were made between VF and non-VF control hearts of both fed ( $n = 10 \times 2$ ) and fasted rats ( $n = 7 \times 2$ ). 6 hearts in fed and 7 in fasted rats were excluded from final analysis due to lack of paired data.

Numbers were entered as follows:



The isolated heart was pre-perfused with 5.5 mM glucose for 20 minutes. Ischaemia was then induced for a maximum of 20 minutes. If VF occurred, 0.5 ml of 1% Evans blue (to identify the ischaemic from non-ischaemic myocardium) was injected through the aortic cannula. The heart was then immediately freeze clamped. At the same time a heart from a rat subjected to the same feeding conditions that did not fibrillate was freeze clamped (also after the Evans blue injection) as a control.

### 5.2.3 The effects of fasting on VF

The effect of 48 hours starvation on ischaemia-induced VF in isolated heart was examined. The hearts were isolated and perfused normoxically with perfusate containing 5.5 mM glucose. A 20 minute period of ischaemia was induced and the incidence of VT and VF was recorded.

#### **5.2.4 The effects of myocardial glycogen on ischaemia-induced VF**

All hearts were pre-perfused normoxically for 20 minutes. Myocardial glycogen levels were raised by perfusing the heart of fasted (48 hours) rats with 15 mM glucose and 2000  $\mu\text{U ml}^{-1}$  insulin. The control hearts of fasted rats were perfused with 5.5 mM glucose without insulin. Hearts were subsequently LAD ligated for 20 minutes before being freeze clamped.

### 5.3 RESULTS

#### 5.3.1 Factors affecting myocardial glycogen and metabolites

The prolonged perfusion for 20 minutes reduced myocardial glycogen and CP levels significantly. However, there was no reduction of myocardial glycogen levels in hyperinsulinaemic or hyperglycaemic hearts. It was noted that insulin or 15 mM glucose perfusion resulted in raised myocardial free glucose levels. There was no correlation between myocardial glycogen levels and free glucose or intracellular glucose levels in any group (Table 5.1).

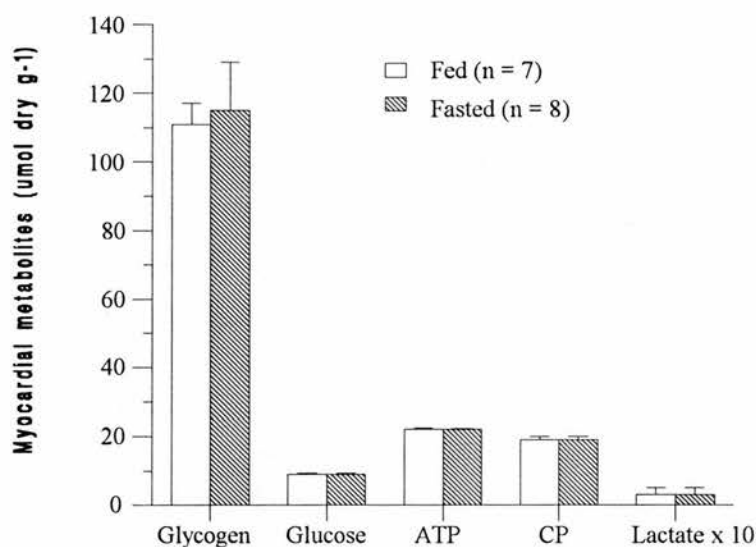
**Table 5.1** Myocardial metabolites in isolated perfused rat hearts

| Myocardial<br>metabolites<br>( $\mu\text{mol g}^{-1}$ dry wt) | Control<br>(n = 7) | Prolonged<br>perfusion<br>(n = 8) | Hyperglycaemia<br>(n = 10) | Hyperinsulinaemia<br>(n = 6) | Hyperglycaemia<br>Hyperinsulinaemia<br>(n = 10) |
|---|--------------------|-----------------------------------|----------------------------|------------------------------|---|
| Glycogen  | 111 (6)            | 76 (5)***                         | 112 (6)                    | 120 (9)                      | 138 (7)***                                      |
| Free glucose  | 9 (0.4)            | 11 (0.2)**                        | 29 (1)***                  | 15 (1)***                    | 47 (1)***                                       |
| Glucose <sub>i</sub>  | 3.4 (0.4)          | 5 (0.2)*                          | 14 (1)***                  | 10 (0.5)***                  | 32 (0.7)***                                     |
| ATP   | 22 (0.4)           | 23 (0.4)                          | 23 (1)                     | 24 (1)                       | 24 (1)  |
| CP  | 19 (1)             | 14 (1)**                          | 17 (1)                     | 20 (1)                       | 20 (1)  |
| Lactate   | 0.3 (0.2)          | 0.5 (0.3)                         | 0.6 (0.3)                  | 0.9 (0.5)                    | 1.2 (0.4)                                       |

The control hearts were clamped after equilibrium perfusion. The experimental hearts were perfused for 20 minutes. The results are expressed as mean (sem) for the number of hearts shown (n). Myocardial glycogen levels in hyperglycaemic vs. hyperinsulinaemic group:  $p = 0.006$ . \*\*:  $p < 0.01$  \*\*\*  $p < 0.001$ , vs. control, t - test. Glucose<sub>i</sub>: intracellular glucose.

The glycogen levels did not differ between fed and fasted rats after 10 minutes equilibrium perfusion (Fig. 5.1)

**Fig. 5.1** Myocardial metabolites of fasted rats after equilibrium perfusion. Perfusate containing 5.5 mM glucose. The experimental hearts came from 48 hours fasted rats. Each column represents the mean with vertical lines indicating s.e.m..



### 5.3.2 Myocardial glycogen and metabolites at the onset of VF in fasted rats hearts

During pre-ischaemic oxygenated perfusion, total coronary flow (area under the curve, ml g<sup>-1</sup> dry wt 20 min<sup>-1</sup>) was not significantly different between control and VF hearts in both fed [897 (69) vs. 855 (25)] and fasted [912 (62) vs. 1051 (72)] rats ( $p > 0.1$ ,  $t$ -test).

The pre-ischaemic total coronary lactate production (area under the curve,  $\mu$ mole g<sup>-1</sup> dry wt 20 min<sup>-1</sup>) was lower in VF hearts of fed rats [control = 49 (7) vs. VF = 29 (6),



$p = 0.048$  t - test]. There was no significant difference in the fasted rat hearts [22 (8) vs. 19 (5),  $p = 0.8$ ].

The reduction in coronary flow after ischaemia did not differ between hearts from fed rats that did or did not fibrillate [control = - 48(3)%, VF = - 39(5)%.  $p = 0.1$ , t - test]. The results were very similar in hearts from fasted rats [control = - 37(2)%, VF = - 35(5)%.  $p = 0.7$ , t - test].

VF occurred after approximately 10 minutes of ischaemia. There was no significant difference in the onset of VF between fed [10 (1) min] and fasted [12 (1) min] ( $p = 0.1$ , Mann-Whitney test) rat hearts. There was no significant difference in the time when hearts were freeze clamped [fed 11 (0.8) min, fasted 13 (1) min]. In the non-VF control hearts, the incidence of VT (fed 9/10; fasted 6/7) was as common as in the hearts in which VF occurred (VT: fed 9/10; fasted 7/7). There was no significant difference ( $p = 0.16$ , Mann-Whitney test) in VF duration between hearts of fed [0.3 (0.1) min] and fasted [0.3 (0.4) min] rats.

In the hearts of fed rats, the ischaemic myocardium of fibrillating hearts had lower myocardial glycogen levels; the non-ischaemic myocardium had higher lactate levels (Table 5.2).

In the hearts of fasted rats, there were no significant differences in any of the myocardial metabolites measured between VF and non-VF control hearts (Table 5.3).

As expected, the ischaemic myocardium had significantly higher lactate levels and lower glycogen and ATP levels than non-ischaemic myocardium (Table 5.2 and Table 5.3).

**Table 5.2** The myocardial metabolites of ischaemic and non-ischaemic areas of fed rats at the onset of VF.

| Myocardial metabolites<br>( $\mu\text{mole g}^{-1}$ dry wt) | Control<br>(n = 10) | VF<br>(n = 10) | t - test<br>p value |
|---|---------------------|----------------|---------------------|
| Glycogen  | 89 (8)              | 85 (6)         | NS                  |
| Glucose   | 15 (4)              | 12 (1)         | NS                  |
| ATP   | 17 (1)              | 17 (0.4)       | NS                  |
| CP  | 6 (1)               | 5 (0.3)        | NS                  |
| Lactate   | 19 (3)              | 26 (2)         | 0.03                |
| Glycogen ischaemic area.                                    | 30 (3)***           | 22 (3)***      | 0.02                |
| Glucose ischaemic area.                                     | 12 (1)              | 10 (1)         | NS                  |
| ATP ischaemic area.   | 8 (1)***            | 8 (1)***       | NS                  |
| CP ischaemic area.  | 2 (0.3)***          | 1 (0.1)***     | NS                  |
| Lactate ischaemic area.                                     | 108 (5)***          | 110 (5)***     | NS                  |

The results are expressed as mean (sem) for the number of hearts shown (n).

\*:  $p < 0.05$ , \*\*\*:  $p < 0.001$  vs. non-ischaemic area, paired t - test.

**Table 5.3** The myocardial metabolites of fasted rats at the onset of VF.

| Myocardial metabolites<br>( $\mu\text{mole g}^{-1}$ dry wt) | Control<br>(n = 7) | VF<br>(n = 7) | t - test<br>p value |
|---|--------------------|---------------|---------------------|
| Glycogen  | 104 (11)           | 103 (6)       | NS                  |
| Glucose   | 11 (1)             | 13 (1)        | NS                  |
| ATP   | 17 (1)             | 16 (1)        | NS                  |
| CP  | 8 (2)              | 5 (1)         | NS                  |
| Lactate   | 13 (3)             | 17 (1)        | NS                  |
| Glycogen ischaemic area.                                    | 39 (8)***          | 29 (2)***     | NS                  |
| Glucose ischaemic area                                      | 11 (1)             | 10 (1)*       | NS                  |
| ATP ischaemic area  | 7 (1)***           | 6 (0.4)***    | NS                  |
| CP ischaemic area   | 2 (0.4)**          | 2 (0.3)*      | NS                  |
| Lactate ischaemic area                                      | 99 (7)***          | 109 (7)***    | NS                  |

The results are expressed as mean (sem) for the number of hearts shown (n).

\*:  $p < 0.05$ , \*\*\*:  $p < 0.001$  vs. non-ischaemic area, paired t - test.

The heart rate [mean (sem) minute<sup>-1</sup>] did not differ between fed and fasted rat hearts both before and after ischaemia [before ischaemia: 275(12) (n=20) vs. 275(13) (n=14), p=1, t-test; ischaemic 5 minutes: 261(11) (n=18) vs. 268(15) (n=14), p=0.7, t-test]. There were no significant difference in the heart rate between VF and non-VF control hearts of both fed and fasted rats (Table 5.4).

**Table 5.4** Heart rates of VF and non-VF control hearts of both fed and fasted rats.

| Heart rate<br>(minute <sup>-1</sup> ) | Fed rats hearts   |                         | Fasted rats hearts |                         |
|---------------------------------------|-------------------|-------------------------|--------------------|-------------------------|
|                                       | Initial<br>(n=10) | Ischaemia 5min<br>(n=9) | Initial<br>(n=7)   | Ischaemia 5min<br>(n=7) |
| Control                               | 270(19)           | 269(20)                 | 250(15)            | 244(21)                 |
| VF                                    | 280(15)           | 252(8)                  | 299(17)            | 292(18)                 |
| t-test (p=)                           | 0.7               | 0.4                     | 0.053              | 0.11                    |

The results are expressed as mean (sem) for the number of hearts shown (n).

### 5.3.3 The effects of fasting on VF *in vitro*

Fasting (48 hours) did not affect the onset of VF or the incidence of ischaemia-induced VF (Table 5.5). The myocardial glycogen levels also did not differ before LAD ligation (Fig. 5.1), although the lactate production was higher in fasted rat hearts before ischaemia (Table 5.6).

**Table 5.5** The incidence of ischaemia-induced arrhythmias (%) in the isolated hearts of fasted rats.

| Arrhythmias    | Control (n = 13) | 48h fasted (n = 17) |
|----------------|------------------|---------------------|
| VT %           | 92%              | 100%                |
| VT onset (min) | 8.8 (0.6)        | 8.9 (0.3)           |
| VF %           | 62%              | 53%                 |
| VF onset (min) | 12.2 (0.7)       | 14.3 (1.2)          |

All hearts were perfused with 5.5 mM glucose. Ischaemia was induced for 20 minutes. The onset of arrhythmias are expressed as mean (sem) for the number of hearts shown (n). There were no significant differences between control and fasted rats.

**Table 5.6** Lactate production before and after LAD ligation.

| Coronary lactate production                        | Control   | 48h fasted |
|--|-----------|------------|
| ( $\mu\text{mol g}^{-1} \text{ dry wt min}^{-1}$ ) | (n = 13)  | (n = 17)   |
| Initial (before ischaemia)                         | 2.1 (0.2) | 3.2 (0.4)* |
| 5 min ischaemia                                    | 4.2 (0.3) | 4.9 (0.3)  |
| 10 min ischaemia                                   | 5.1 (0.5) | 5.9 (0.4)  |

Hearts are the same as those of Table 5.5. The results of the coronary lactate production after 10 minutes of ischaemia were not given, as VF had started. The results are expressed as mean (sem) for the number of hearts shown (n).\*:  $p < 0.05$  vs. control, t - test.

Ischaemia induced a 39 - 41% fall in coronary flow in hearts. There were no significant differences in the flow reduction either between the hearts of fed and fasted rats or between the hearts of non-VF and VF group (t - test).

#### 5.3.4 The effects of myocardial glycogen on ischaemia-induced VF

The high glycogen (or hyperglycaemia-hyperinsulinaemia) hearts had 81% higher glycogen levels than control (prolonged perfusion) hearts (Table 5.1). However, the increase in myocardial glycogen did not result in a reduction in the incidence of VF [control hearts: 7/11 (64%), in high glycogen hearts: 11/12 (92%).  $p = 0.3$ , Fisher's exact test, two side probability]. All hearts developed VT at approximately 8 minutes after coronary artery ligation. There were no significant differences in the onset of VT in control vs. high glycogen hearts [8 (0.3) vs. 8 (0.7).  $p = 0.6$ , Mann-Whitney test] or in the onset of VF [11 (1) in control vs. 11 (1) minutes in high glycogen hearts.  $p = 0.9$ , Mann-Whitney test].

The reduction in coronary flow rate due to LAD ligation were not significantly different between the hearts that did or did not fibrillate ( $p = 0.55$ ,  $t$  - test). There was also no difference in the reduction in coronary flow between glycogen loaded and control hearts:  $-25(3)\%$  vs.  $-28(3)\%$ .

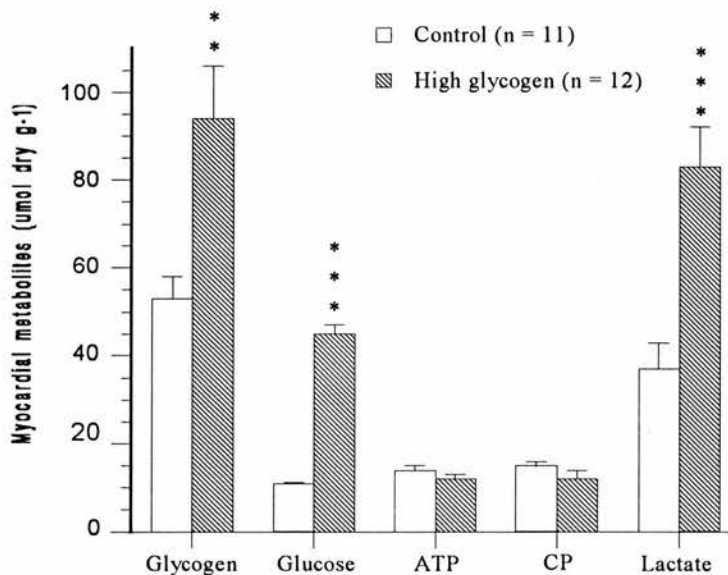
The total lactate production during pre-ischaemic perfusion [area under the curve, mean (sem)  $\mu\text{mole g}^{-1}$  dry wt 20 minutes $^{-1}$ ] was higher ( $p < 0.0001$ ,  $t$  - test) in glycogen loaded [68 (6)] than control hearts [5 (3)] as expected.

The coronary lactate production during ischaemia (area under the curve,  $\text{ml g}^{-1}$  dry wt 20 min $^{-1}$ ) did not differ ( $p = 0.08$ ,  $t$  - test) between control [930 (66)] and high glycogen [783 (32)] hearts.

The levels of myocardial glycogen in glycogen loaded hearts were still higher after 20 minutes ischaemia. However this did not result in higher CP or ATP levels (Fig.5.2).

**Fig. 5.2** Myocardial metabolites after 20 minutes coronary artery ligation.

Each column represents the mean with vertical lines indicating s.e.m.. \*\*,  $p < 0.01$ , \*\*\*,  $p < 0.001$  vs. control,  $t$  - test.



## 5.4 DISCUSSION

It cannot be assumed that myocardial glycogen levels will remain constant during normoxic perfusion. In fact, myocardial glycogen ( $p < 0.001$ ) and CP ( $p < 0.01$ ) levels fall after prolonged perfusion with 5.5 mM glucose (Table 5.1). The increased free glucose ( $p < 0.01$ ) in the hearts is a result of increased glycolysis and increased glucose uptake.

The mechanism of the decline of myocardial glycogen after prolonged perfusion could be related to slow wash out of myocardial insulin (see Chapter 1 for review). Insulin is responsible for passive glucose uptake and energy dependent glucose transport. The passive glucose uptake is also glucose concentration dependent (Chapter 1). This is the reason that the decline in glycogen levels can be prevented by adding insulin to the perfusate or by increasing perfusate glucose levels (from 5.5 mM to 15 mM) (Table 5.1). Both insulin and higher glucose levels (15 mM) raise myocardial intracellular glucose significantly. The latter method raises intracellular glucose to a greater degree than the former ( $p = 0.006$ ). This suggests that insulin affects glycogen formation while higher extracellular glucose only enhances glucose uptake.

The results have shown that despite an almost doubling of the myocardial glycogen content, the ATP levels at the end of a 20 minute ischaemic period almost always remained unchanged, as did those of CP (which is more sensitive indicator of energy reduction than ATP). This clearly demonstrates that myocardial glycogen did not produce sufficient ATP to prevent the ischaemia-induced loss in high energy phosphates. We were unable to determine whether the initial rate of ATP loss had been reduced.

The results for the myocardial metabolites at the onset of VF showed that myocardial glycogen levels, or the differences in the glycogen levels between ischaemic and non-ischaemic area cannot predict which heart will fibrillate and which will not.

There were no significant difference in heart rate between VF and non-VF control hearts of both fed and fasted rats (Table 5.4). In patients with unstable angina, ischaemia of the anterior wall is coupled with a marked increase in heart rate, which precedes the onset of 42% VT and 37% VF. On the other hand, patients with inferior ischaemia show a reduction in the heart rate and infrequent onset of VT (8%) and VF (3%) (Perez-Gomez, *et al.*, 1979). Similarly, increase in heart rate during ECG monitoring precedes VF in patients with acute MI (Adgey, *et al.*, 1982, McAreavey, *et al.*, 1989).

In the fed rat hearts, the VF hearts had lower myocardial glycogen levels in the ischaemic area and higher lactate levels in the non-ischaemic area when compared with non-VF control. This suggests a higher glycolysis rate in the VF hearts. In the ischaemic myocardium, the higher glycolysis rate resulted in an increased glycogen consumption which cannot be replaced by perfusate glucose. In the non-ischaemic myocardium, the tissue glycogen levels were not reduced as the myocardium is still supplied with external glucose. This led to an increase in tissue lactate levels. At the onset of VF, the ATP levels did not differ between VF and non-VF control hearts. This indicates that ATP production relates to its consumption. The data suggests that a heart which consumes more ATP during ischaemia is more likely to develop fibrillation. The differences in glycogen levels of ischaemic myocardium between the VF and non-VF hearts did not result in a different myocardial ATP levels up to the point of VF (Table 5.2). The tissue lactate levels in the ischaemic myocardium did not differ between VF and non-VF control hearts. This does not support the view that the acidosis is the main cause of VF (Table 5.2 and Table 5.3).

Nevertheless, the data suggests that the heart responds to ischaemia in a different manner. When the levels of pre-ischaemic myocardial glycogen are identical, a

lower glycogen utilisation or the lower rate of glycolysis during regional ischaemia can protect the heart from VF, or can delay the onset of VF as the control hearts may develop VF later. This may be more important to the ischaemia-induced VF than acidosis induced damage or than glycolytic ATP production.

In the fasted rat hearts, however, the lower myocardial glycogen levels in the ischaemic area and higher tissue lactate levels in the non-ischaemic area of the VF hearts were not significant.

Fasting is one of the common methods used to increase myocardial glycogen levels. Schneider and Taegtmeyer (1991) reported that fasting improves recovery of function and lessens membrane damage after 15 minutes total ischaemia in isolated working rat hearts. They found that the myocardial glycogen content of rat was raised by 25% after 16 hours overnight fasting. They concluded that the protective effects were due to increased glycogen levels by fasting prior to ischaemia.

It was therefore expected that fasting might protect myocardium against the development of ischaemia-induced VF *in vitro* due to increased myocardial glycogen *in situ*. However, the present study does not support this hypothesis. The possible explanation is that the increased glycogen had returned to control level after equilibrium perfusion. A possible protective factor of the higher glycogen was therefore removed, which means that fasting itself cannot protect the heart from ischaemia-induced VF. The results also suggest that the isolated perfused heart is preferentially using glycogen rather than glucose when glucose is the sole substrate.

To avoid the depletion of myocardial glycogen in fasted rat heart, an *in vivo* experiment could be used. However, the influence of general anaesthesia, and systemic NA effects on cardiac glycogen need to be taken into consideration.



Fasted rat hearts were perfused with 15 mM glucose and 2000  $\mu\text{U. ml}^{-1}$  insulin before LAD ligation to ensure myocardial glycogen levels were higher than in fed controls. Even in these extreme circumstances increased myocardial glycogen could not protect the heart against ischaemia-induced VF.

Myocardial glycogen and free glucose levels were still higher in the experimental hearts after 20 minutes of ischaemia. However, there were no significant differences in ATP and CP levels when compared with fed controls (Fig. 5.2). The higher glycogen content is the result of increased glycogen synthesis in the heart. The heart with higher glycogen content did not produce extra ATP from glycogen during ischaemia as the lactate production was not significantly lower than control ( $p = 0.08$ ). The data suggests that loading glycogen with insulin and high glucose may inhibit rather than promote glycolysis. The precise information of what happens in ischaemia area should be obtained by measuring tissue metabolites of ischaemic and non-ischaemic myocardium separately in future studies.

These results suggest that increased glycogen before the onset of ischaemia does not necessarily improve energy supply during ischaemia. That is, the energy production of the heart during ischaemia is not necessarily dependent on the content of myocardial glycogen.

Although there was not a significant difference in the incidence of VF between glycogen loaded and control hearts (92% vs. 64% in control,  $p = 0.26$ ), the magnitude of this difference is clinically important. Knowing the incidence of VF, it is now possible to calculate the number of animals required to document this with a power of 90% at 5% level of significance. This requires two groups of 40 rats.

## 5.5 SUMMARY

1. The higher myocardial glycogen levels induced by prolonged fasting returned to basal levels after a 10 minute equilibrium perfusion. This should be taken into consideration when studying the effects of any *in vivo* condition in an isolated rat heart preparation.
2. Fasting itself does not affect the incidence of ischaemia-induced VF of the hearts *in vitro*, when basal glycogen levels between fed and fasted rat hearts are the same before ischaemia.
3. Increasing myocardial glycogen by perfusing hearts of fasted rats using a buffer high in glucose (15 mM) and insulin also did not affect the incidence of ischaemia-induced VF *in vitro*.
4. Myocardial glycogen levels in the ischaemic area of fibrillating hearts of fed rats were lower than the levels in those hearts that did not fibrillate. Lactate production was higher in the non-ischaemic area. This suggested a higher glycogen utilisation or glycolysis rate in the VF hearts. This might have contributed to the development of the VF.

**In conclusion:** The method of raising myocardial glycogen levels or the rate of glycolysis in the heart is more important than the glycogen level itself to protect the heart against the development of ischaemia-induced VF.

## **CHAPTER 6**

### **General Discussion**

## 6.1 THE EFFECT OF MYOCARDIAL GLYCOGEN IN THE ISCHAEMIC HEART

### 6.1.1 Glycogen and arrhythmias

The aim of the study was to examine the role of myocardial glycogen in the ischaemic heart. Fasting has been shown to be beneficial to myocardial contractile recovery post-ischaemia. The mechanism is thought to be due to increased myocardial glycogen content prior to ischaemia. The effects of fasting *per se* on ischaemia-induced arrhythmias have been examined (Chapter 5). When the differences in myocardial glycogen levels between fed and fasted rats were removed, there were no significant differences in the incidence of ischaemia-induced arrhythmias.

Myocardial glycogen levels of the hearts of fasted rats were also raised by glucose and insulin perfusion. Insulin itself was expected to protect the heart from arrhythmias as it activates the sodium pump in the cell membrane (Ewart and Klip, 1995). The inhibition of the pump is arrhythmogenic, this is a well known pharmacological explanation for the toxic effects of digitalis, a sodium pump inhibitor. However, raised glycogen in fasted rat hearts still did not protect the hearts from ischaemia-induced arrhythmias. The reason could be that the ischaemic injury was very severe. Glucose infusion, therefore, promotes rather than prevents arrhythmogenesis (Liedtke *et al.*, 1976; Russell and Oliver, 1977).

In the paired clamped hearts study (Chapter 5), the VF hearts (fed rats) had lower myocardial glycogen content in the ischaemic area, and higher tissue lactate in the non-ischaemic area than non-VF control hearts (Chapter 5). This suggests a higher glycolysis or glucose utilisation in the VF hearts. The lowering of glycolysis rate or glucose utilisation may not be able to prevent but only to delay the onset of arrhythmias as the control hearts may develop VF later.

Schaefer and Ramasamy (1997) recently compared the glycogen utilisation in the isolated rat heart during no-flow global ischaemia. They found fasted rat heart had greater glycogen utilisation during ischaemia than glucose and insulin perfused hearts of fed rats. They concluded that fasting protects the heart from ischaemic injury and is associated with increased glycogen utilisation during ischaemia. Increasing glycogen levels in hearts of fed rats using insulin limits glycogen utilisation, increases ischaemic injury, and impairs ischaemic injury recovery. The ischaemia-induced VF study (Chapter 5) does not support the theory that increased glycogen utilisation can prevent VF.

These data indicate that acceleration of glycogenolysis or enhanced glycogen utilisation cannot protect the heart from ischaemia-induced VF, but is beneficial for post-ischaemia functional recovery. These results also suggest that the different methods used to change the myocardial glycogen level have different metabolic effects on the heart during ischaemia, which may be more important than the glycogen level itself.

#### **6.1.2 Myocardial glycogen on anoxia induced noradrenaline release**

Insulin has been shown to decrease noradrenaline overflow during anoxia (Chapter 3). This result reflects what happens in the centre of the ischaemic area, especially in the early stage of ischaemia. However, the results cannot be applied to ischaemia directly. In anoxic hearts, coronary flow was not restricted. Increasing myocardial glycogen levels before ischaemia using a similar method (as in Chapter 3) did not protect the heart from VF (Chapter 5). This evidence suggests that it is acidosis, not noradrenaline overflow, that contributes to ischaemia-induced VF. On the other hand, in regional ischaemia, the acidosis (Haass, *et al.*, 1990; Miyazaki and Zipes, 1990) and increased extracellular concentration of  $K^+$  (Forfar and Riemersma, 1987; Miyazaki and Zipes, 1990) could inhibit noradrenaline overflow, which

could cause noradrenaline to be less important in generating arrhythmias during regional ischaemia.

Insulin decreases anoxic noradrenaline overflow in the low  $K^+$  perfused heart (Chapter 3). This result has an experimental significance. In arrhythmia studies the isolated rat heart is often perfused with low potassium to induce a high incidence of arrhythmias. However, the result does not have clinical application as in hypokalaemia, the treatment is to administer potassium rather than insulin. Insulin on the other hand is often used to treat hyperkalaemia as it transfers potassium into the cell.

There is an advantage in taking multiple samples for lactate production during anoxia. The highest lactate production was at 1 to 2 minutes after the onset of anoxia (Chapter 3). This provides more detailed information on glycolysis when compared with the previous work in our department (Dart *et al.*, 1987). In fact the glycolytic rate is increased for only several minutes after the onset of ischaemia (Kubler and Spieckermann, 1970; Rovetto *et al.*, 1973) followed by inhibition due to intracellular acidosis (Neely *et al.*, 1975; Rovetto and Neely, 1979). In the dog, accelerated myocardial glycolysis appears less than 10 seconds after perfusion has stopped (Jones *et al.*, 1976). By 30 seconds after the onset of ischaemia, the metabolic flux through glycolysis has become marked compared to the normal state. Within 2 - 3 minutes of ischaemia, the rate of glycolysis is only one quarter of the maximum value. However, this less effective glycolysis will still continue for about 30 minutes in the area of total ischaemia (Jones *et al.*, 1976).

Myocardial glycogen levels, ATP and CP during anoxia were not measured, but during glucose free anoxia, ATP can only come from glycolysis. This can be indirectly measured by lactate production. It is known that ischaemia causes a marked depletion of CP and ATP. The myocardial CP level falls by over 80% within 2 - 3 minutes in ischaemia, with ATP content declining more slowly (Alpert, 1989). CP is therefore a more sensitive

parameter than ATP. The function of CP is to act as a store of high energy phosphate to maintain ATP concentrations in muscle when ATP is being rapidly utilised as a source of energy for muscular contraction. From the lactate production it can be observed that the rate of anaerobic glycogenolysis declines long before glycogen is depleted. This is in agreement with the earlier findings by other researchers (Opie, 1968; Cornblath *et al.*, 1963).

## 6.2 METHODOLOGY

Starvation often faces patients clinically, as patients in acute pain generally do not want to eat. In addition, patients are often advised not to eat pre-operatively to reduce the chance of a blocked airway due to vomiting during surgery. Schneider and Taegtmeyer (1991) found that 16 hours overnight fasting increased myocardial glycogen content by 25% in male Sprague-Dawley rats. The study in Chapter 4 showed that myocardial glycogen levels did not increase after 24 hours fasting, but after 48 hours fasting they did increase.

The differences between these two studies could be due to the diurnal variations influencing myocardial glycogen concentrations in the hearts of rats (Asimakis, 1996; Segel *et al.* 1975). The cardiac glycogen level reaches its peak in the morning and is lowest in the evening (Conlee *et al.*, 1976; Bockman *et al.*, 1971; Segel *et al.*, 1975). In Sprague-Dawley rats, the difference can be as much as 20% (Daw, 1969). It is believed that the changes are related to the diurnal fluctuations in plasma corticosteroid concentration (Daw, 1969). In addition, diurnal rhythm is often controlled by the artificial light in animal houses. The period of lighting could also affect myocardial glycogen levels. Diurnal effects on myocardial glycogen levels in fasted rats have not previously been studied.

In the perfusion study, myocardial glycogen levels raised by fasting can diminish to those of the controls by the end of 10 minutes perfusion with 5.5 mM glucose (Chapter 5). The main reason for the reduction could be that fasted animals have lower plasma insulin levels, which cause decreased glucose transport. Insulin remains bound to tissue when the rat heart is isolated. The bound insulin affects glucose uptake or transport for up to 30 minutes after commencing perfusion (Fisher and O'Brien, 1972). Accordingly, 30 minutes pre-perfusion is required to reduce glucose transport to a basal level (Zachariah, 1961; Mansford, 1967). This should therefore be taken into consideration when considering insulin's effects.

The study of factors affecting myocardial metabolites (Chapter 5) has shown that the levels of CP and glycogen were decreased after 20 minutes prolonged normoxic perfusion. This suggests a low oxygen capacity of perfusate as haemoglobin is absent. Even with perfusate  $pO_2$  as high as 543 mmHg and with high flow rates (Chapter 2), the oxygenation of the tissue is still limited. The model is thus more useful for studying the effects of ischaemia rather than being used as a model of normoxic metabolism.

### 6.3 LIMITATIONS OF THE STUDY

In the anoxia study, normal flow was used to measure noradrenaline overflow during the 40 minutes of anoxia. The data can not be applied directly during ischaemia. However, some of the early changes during anoxia or ischaemia are similar.

Due to the limitations of costs, time, model setting and laboratory facilities, it was not possible to measure some important parameters. It is not possible to measure the glycogen levels in the nerve endings in the heart. The controls of the study in Chapter 4 were not ideal. Some



of the conclusions depend in part on data from others. i.e. the plasma insulin levels during anaesthesia (Chapter 5), and therefore remain somewhat speculative.

The glucose uptake by the heart was not measured, as the amount of glucose taken up by the heart is very small compared with the plasma glucose concentration. Measurement of glucose uptake is therefore subject to large errors and its estimation is limited by the precision of the glucose assay being used (Riemersma, 1979). Samples must be analysed with an extremely high precision or numerous times in order to obtain accurate results. Alternatively, a recirculating system needs to be used. As the main hypothesis was directed at the effects of myocardial glycogen, the simpler non-recirculating isolated rat heart model was used (Fisher and O'Brien, 1972).

Myocardial glycogen concentration declined after 20 - 30 minutes perfusion with 5 mM glucose (Chapter 2 and 5). Although the glucose uptake and tissue insulin levels were not measured, the reduction of glycogen levels has been shown to be related to decreased glucose uptake and tissue insulin. The decline of glycogen can be prevented by perfusing the heart with insulin (Chapter 5). Increasing the concentration of glucose to 11 mM in the perfusate did not prevent this decline (Chapter 2), but 15 mM glucose perfusion did prevent the reduction of glycogen (Chapter 5).

#### **6.4 CLINICAL SIGNIFICANCE OF MYOCARDIAL GLYCOGEN STUDY**

The dual roles of myocardial glycogen in the development of ischaemic damage are generally recognised. Increased myocardial glycogen protects the heart from damage by moderate ischaemia. The GIK treatment also decreases the infarct size in dogs (Opie, 1975). It may be that the effect of GIK on infarct size is limited to the area of moderate ischaemia. However pre-increased glycogen cannot reduce ischaemia-induced VF (Chapter 5). Both

Neely and Oliver's team have found that, under very severe ischaemic injury, glucose infusion appears to promote rather than prevent arrhythmogenesis. They conclude that this may be a result of earlier inhibition of glucose utilisation during very severe ischaemia due to more rapid development of intracellular acidosis (Liedtke *et al.*, 1976; Russell and Oliver, 1977).

Coronary heart disease in man is often caused by regional ischaemia rather than global ischaemia. In regional ischaemia, the centre of ischaemic myocardium is more likely to have severe ischaemia or anoxia. Therefore, to avoid the 'side effects' of glycogen loading, GIK should be administered *after* ischaemia. This should prevent glycogen induced acidosis in the severely ischaemic area due to flow restriction. On the other hand, the uptake of glucose in the moderately ischaemic area will be increased, i.e. in the border of the regional ischaemic area, or during reperfusion. The heart should benefit from increased glycolytic ATP, resulting in improved functional recovery. It has been shown that when GIK was given within the first hour of coronary arterial ligation, tissue contents of high energy phosphate compounds in the infarcted myocardium were improved (Opie 1975). The study in dogs has shown that GIK infusion over 6 hours, increased tissue glycogen in all zones, ATP and CP in the infarcted zone, and improved potassium/sodium ion ratio, which is an index of the degree of necrosis. A recent study in rats demonstrated that the effect of GIK on infarct size can be achieved when GIK is administered after reperfusion (Jonassen *et al.*, 1996). GIK infusion has also been beneficial in patients after cardiac surgery (de Villalobos and Taegtmeier 1995).

Several controlled clinical trials have examined the effect of GIK on myocardial infarction, including the MRC's multi-centre study (1968) and clinical research by Mittra (1965). None of them took the time of GIK administration into account. This may be the reason why their results did not agree with each other, although similar protocol was used.

## 6.5 SUGGESTIONS FOR FURTHER RESEARCH

### 6.5.1 Fasting and arrhythmias

The increased myocardial glycogen content after fasting returns to normal within 10 minutes of pre-perfusion with 5 mM glucose (Chapter 5). Further studies, to investigate the effects of fasting on ischaemia-induced arrhythmias, could increase perfusate glucose concentration to 15 mM, in order to maintain the initial myocardial glycogen levels (Chapter 5) before LAD ligation.

### 6.5.2 Glycogen and ischaemic injury

Prior transient episodes of ischaemia ("ischaemic preconditioning") can exhaust myocardial glycogen stores. This reduces lactate accumulation and attenuates acidosis during a subsequent prolonged ischaemic insult. However, whether this is the reason that ischaemic preconditioning improves post ischaemic recovery is still in debate. There have been no experiments designed to test this hypothesis. Further study could consider increasing myocardial glycogen (i.e. with GIK) after a prior transient episode or episodes of ischaemia and before a subsequent prolonged ischaemic insult.

Weiss *et al.* (1996) recently suggested that the protective effect of preconditioning on the ischaemic heart is not the result of a reduced glycogen level, which contributes to decreased lactate accumulation after preconditioning, but due to reduced glycogenolysis. The paired clamped hearts study (Chapter 5) has shown an increase in glycolysis or glucose utilisation

in the fibrillating hearts. However the effect of preconditioning on ischaemia-induced arrhythmias has not been investigated in this thesis.

Animal experiments often represent extreme situations and the therapeutic use in man of agents to modify the metabolism of glucose after myocardial ischaemia needs intensive clinical investigation before general application. Measuring myocardial glycogen content and ischaemic severity using non-invasive techniques would aid our understanding of whether the dose response of GIK differs between patients with mild, moderate or severe ischaemia.

### 6.5.3 Glycogen and arrhythmias

One of the critical factors in the genesis of early ischaemic and reperfusion VF is the rapid development of electrophysiological inhomogeneities within regionally ischaemic areas of myocardium (Janse, 1986; Russell, 1981). The inhomogeneities could to a large extent be determined by similar inhomogeneities of glucose-glycogen utilisation during early ischaemia according to Russell and colleagues (1986). They found that the inhomogeneities of the patterns of conduction within ischaemic areas are accompanied by marked inhomogeneities of glycogen, lactate and ATP distributions and that inhomogeneities of glycogen distribution were greater in hearts that fibrillated than in those that did not.

Pre-conditioning may decrease the inhomogeneities of glycogen and lactate distributions within the ischaemic areas. To administer GIK after the onset of ischaemia can enhance this effect by decreasing the inhomogeneities of myocardial metabolites between the ischaemic zone and the centre of ischaemic area. This could reduce the arrhythmias induced by regional ischaemia. In fact, in the original study of anti-arrhythmic effects of GIK, demonstrated by Sodi-Pallares and colleagues (1963), the GIK was given to the dog *soon after* the coronary artery occlusion.

## 6.6 SUMMARY

1. Myocardial glycogen content in anaesthetised rats can be affected by the anaesthetic used. Pentobarbitone is suitable for studying myocardial glycogen metabolism in fasted rats, provided intermittent hypoxia during anaesthesia is prevented. Extra oxygen supply during anaesthesia may be used in order to minimise myocardial glycogen variability *in vitro*. This may help to reduce group size (Chapter 4).
2. Respiratory acidosis caused by extra oxygen supply during anaesthesia did not appear to have any effect on myocardial glycogen concentrations (Chapter 4).
3. The free glucose in the heart should be allowed for when calculating myocardial glycogen levels, especially when different glucose levels in the perfusate are used within the same experiment (Chapter 2).
4. Insulin prevents anoxic noradrenaline overflow in low potassium perfused hearts, but not in normal potassium perfused hearts (Chapter 3).
5. The higher myocardial glycogen levels induced by prolonged fasting returned to basal level after a 10 minute equilibrium perfusion. This should be taken into consideration when studying the effects of any *in vivo* condition in an isolated rat heart preparation (Chapter 5).

6. Fasting itself does not affect the incidence of ischaemia-induced VF of the heart *in vitro*, when basal glycogen levels between fed and fasted rat hearts were at the same levels before ischaemia (Chapter 5).
7. Increasing myocardial glycogen by perfusing hearts of fasted rats using a solution high in glucose (15 mM) and insulin also did not affect the incidence of ischaemia-induced VF (Chapter 5).
8. Myocardial glycogen levels in the ischaemic area of fibrillating hearts of fed rats were lower than those of hearts that did not fibrillate. Lactate production was higher in the non-ischaemic area. This suggests a higher glycogen utilisation or glycolysis rate in the VF hearts. This might have contributed to the development of the VF (Chapter 5).

The results in this thesis suggest that the method used to increase myocardial glycogen concentration is more important than the glycogen level itself in protecting the heart from ischaemia-induced arrhythmia.

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